1975

Studies On An Early Physical Change In L-m Cells Induced By Infection With Vaccinia Virus

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STUDIES ON AN EARLY PHYSICAL CHANGE IN L-M CELLS INDUCED BY INFECTION WITH VACCINIA VIRUS

by

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Submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario

November, 1974

To Judy and Joshua

ABSTRACT

Density gradients of Ficoll (a high molecular weight synthetic polymer of sucrose) are known to be effective in separating a non-homogeneous cell population (into cell types) and a homogeneous cell population - for example a cell strain (into cells in different phases of the eucaryotic cycle). The success achieved with such gradients, especially in the latter application, suggested the possibility that Ficoll density gradients might have discriminatory properties great enough to at least partly separate virus-infected cells from uninfected cells.

The present study shows that by 2 hours post infection (PI), L-M strain cells infected with vaccinia virus show an alteration in their sedimentation distribution pattern, when subjected to a 15 minute centrifugation at 1000 X g in a 14%-18% (w/v) discontinuous Ficoll gradient. The alteration takes the form of a shift of the cells towards less dense regions. The extent of this shift is both time-dependent and multiplicity of infection (MOI)-dependent, and is maximized at 2-3 hours PI using a MOI of 10. The biophysical basis for the phenomenon appears to be a buoyant density decrease of cells after infection.

Cell size analyses of total populations (infected and uninfected), using a Celloscope electronic cell counter-pulse height channel analyzer system, show that the mean cell volume of a population of cells increases by 13% by 3 hours PI using a MOI of 10. Qualitative cell size data, which supported this finding, was also obtained using a Cytograf laser beam diffraction apparatus.

To gain some understanding of the biochemical events occurring in infected cells which are responsible for the acquisition of this altered sedimentation property by the L-M cells, a series of experiments was performed in which either DNA synthesis, RNA synthesis or protein synthesis was inhibited by the addition of an appropriate antimetabolite. It was found from these studies that DNA synthesis was not required, but that RNA and protein syntheses were required, in order that the altered sedimentation property be acquired. The results which were obtained in this study were not sufficient to allow a differentiation to be made between the possibilities that the genetic information coding for the required RNA and protein species resided in the viral genome or in the cell genome. However, the results obtained in this study and others, in which cells were infected with vaccinia virus inactivated either by heat treatment or ultra-violet irradiation, suggests that it is the viral genome which contains this information.

Assuming that the information which must be expressed for the acquisition of the altered sedimentation property resides in the viral genome, the RNA and protein species which are required can be described as "early" molecules on two bases: first, from a temporal standpoint, since the sedimentation shift occurs by 2-3 hours PI, which is at least 6 hours prior to completion of synthesis of progeny virions; and second, from a biochemical definition of "early" as any species of molecule synthesized prior to replication of parental viral DNA. Furthermore, since no deoxyribonuclease-sensitive (released) parental viral DNA was detected in infected cells for up to 4 hours PI, the required RNA and protein species might further be classified as "early early", that is synthesized prior to secondary uncoating, while the parental viral DNA is still associated with the core. To date the only vaccinia virus molecules which have been shown to be synthesized during the "early early" period are thymidine kinase and the putative uncoating protein.

It is likely that eventually, with time after infection, every cell in a population can acquire the altered sedimentation property. Cells in the G2 phase of the cell cycle at the time of infection may possibly contribute to the shifted population by 3 hours PI, whereas cells in S phase at the time of infection apparently make their contribution later (between 3 and 8 hours PI). Thus

the phenomenon may be time-dependent for 2 reasons: 1) Vaccinia virus replication within infected cells must reach a specific stage (between primary and secondary uncoating), at which the required RNA and protein species are synthesized; and 2) Vaccinia virus replication may take longer in some cells than in others, reflecting a possible dependency of replication on the eucaryotic cell cycle.

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank the following individuals for their help to me over the past four years, while working on this project:

First I would like to express my thanks to my supervisor, Dr. E. L. Medzon, for the patience and understanding he displayed throughout this study, and for his confidence in me. His guidance in the way of discussions, suggestions, and criticisms of my work proved to be invaluable.

To my laboratory colleagues, Mr. Tekena Harry, Mr. Jim Faught, and Mr. Geoffrey Dilworth, my thanks for their co-operation, interest in my work, and their companionship. I would also like to acknowledge with thanks, the helpful discussions at the outset and conclusion of this project with Dr. Margaret Creighton, who laid so much of the groundwork on which this thesis was built. Special thanks are due to Dr. Lampros Jakovidis for capably assuming the technical aspects of running this laboratory. I would be remiss if I did not gratefully acknowledge the guidance given to me at the beginning of this project by Mr. Adolph Gedies, who introduced me

to the techniques of tissue culture and the "world of sterility".

It is my pleasure to acknowledge the kind co-operation displayed to me by Drs. I. G. Walker, W. P. Cheevers, and A. Day in permitting me on numerous occasions to freely use apparatuses in their laboratories. I also sincerely thank Mr. Tim Bowden for help with the Celloscope cell sizing experiments.

A tremendous debt of gratitude is owed to the entire Health Sciences Library staff, and in particular Mrs. Agnes Kutas, for their utmost co-operation during this project, in helping me solve my library-oriented problems.

In the interests of space I shall refrain from singling out individual members, but I would sincerely like to thank those faculty, staff, and students of the Department of Bacteriology and Immunology who have helped make my stay in this department a truly enjoyable one. Special thanks go to Mr. George Sanders for all his help.

Special thanks are also due to Mrs. Sandy Davy and Mrs. Irene Tomlin for their co-operation and patience, and for their expert and efficient typing and printing of this manuscript.

I would like to express my appreciation to Drs. M.M. Ali, M.S. Haq, and S. Sangal, for their help in recommending the statistical approaches to use in treating my data.

Last, but by no means least, I thank my wife Judy. Her patience and understanding of the problems which beset graduate students have been very much appreciated. Her unwavering confidence in me over the years has spurred me on in the face of setbacks and disappointments. My indebtedness to her is immeasurable.

This work was supported in part by an operating grant to Dr. E.L. Medzon by the Medical Research Council of Canada.

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INTRODUCTION

A study and appreciation of the mammalian cell cycle leaves one with a certain "attitude" about tissue culture cells; that, although they co-exist and indeed co-operate in culture very well, they are not equal in all respects. This "inequality" is often the result of the fact that cells in a logarithmically growing population are in different phases of the cell cycle at any one time. One way in which this inequality might express itself is in terms of sensitivity to virus infection.

A close monitoring of the literature over the past number of years has revealed to this investigator that evidence is accumulating which indicates that, although many cell-virus systems differ in terms of which cell cycle phases virus replication is dependent on, a general theme emerges: viz. the mammalian cell cycle DOES play a significant role in virus replication.

This observation suggested the possibility of separating virus-infected cells from uninfected cells, for it has been known for some years now that cells from a log phase population can be separated from each

other on the basis of the phase of the cell in its traverse around the cycle. Studies which separated malignant from non-malignant cells in density gradients suggested that efforts with virus-infected cells might prove successful.

The choice of vaccinia virus as the model virus for this study seemed logical in view of the fact that so much information was already available on the replication of this virus. Choice of the L-M cell, a derivative of strain L cell, as the model host cell also seemed logical since its growth properties were familiar and well studied in this laboratory.

This study deals with the application of Ficoll density gradients to separate infected from uninfected cells, and attempts to explain the mechanism and basis for the separation. Ficoll gradients were used strictly as an analytical tool, and never as a preparative tool, to ensure that cells being studied were in as close to a "normal" state as possible. Although Ficoll is accepted to be a good material to use for gradient centrifugation of cells, the danger always exists that cells recovered from a Ficoll gradient have been altered in some way due to exposure to this substance.

HISTORICAL REVIEW

A. CELL

The cell strain used exclusively in this study was L-M (American Type Culture Collection CCL 1.2). It was derived from NCTC clone 929 by Kuchler and Merchant (1956), this clone in turn being derived in 1948 from the parental strain L. Strain L cell was obtained from normal mouse connective tissue and today is noted as one of the first to be established in continuous culture (ATCC Registry, 1972).

Strain LM cells are characterized by their propagation in serum-free medium 199 (Morgan, Morton, and Parker, 1950). Morphologically, they are fibroblast-like when grown in monolayer and karyotypically are heteroploid, with 62 chromosomes per cell being the most frequent number encountered.

There are at least two advantages in using this particular cell strain. First is the ability of L-M cells to grow well in both monolayer and suspension cultures. Furthermore these cells can easily be transferred from a monolayer culture to a suspension culture, and vice-

versa (see Materials and Methods).

A second advantage of using the L-M cell is that it grows well in the absence of serum. For a number of reasons, we feel it is highly beneficial to avoid the use of serum. First there is the constant worry of contamination of the cell culture with mycoplasma or other organisms, the source of which in many cases has been traced directly to the serum. Secondly, by omitting serum from medium 199 we have, with the exception of the 0.5% peptone additive (Eidam and Merchant, 1965), a completely defined medium. Finally, since serum is known to affect cell membranes (Castor, 1971; Witkowski and Brighton, 1972), it is advisable to omit serum in studies on possible cell membrane changes.

The ability of L-M cells to support the replication of a wide range of viruses in our laboratory (mengovirus, reovirus, vaccinia virus, equine herpesvirus, vesicular stomatitis virus, and others) can be considered as an additional advantage, especially for comparative work (Dr. L. Jacovidis, personal communication).

Finally, depending on one's point of view, it can be considered to be an advantage that the L-M cell grows with a relatively long doubling time (approximately 35 hours) in this laboratory in suspension culture. This

lengthy cell cycle is advantageous in that it increases the likelihood of *selecting* out sub-populations, from asynchronously growing cultures, which are homogeneous with respect to the stage of the cells in their traverse around the cycle; in other words the longer a cell spends at each stage in the cycle (G1, S, G2, M) the greater the chance of isolating that cell while still in that particular stage, since even the most rapid method of isolation takes considerable time. The long doubling time is a distinct disadvantage, however, if one is attempting to *induce* synchrony in an asynchronous population, since the longer the doubling time the longer the inducing agent must be present in the culture to arrest all cells at a particular point in the cycle.

As alluded to above, the L-M cell in an exponentially growing suspension culture cycles around the typical eucaryotic cell cycle, going through the following stages (Howard and Pelc, 1953):

1) G_1 - the post-mitosis and pre-DNA synthesis stage;
2) S - the DNA synthesis stage. Here the DNA content of the cell doubles in preparation for mitosis;
3) G_2 - the post-DNA synthesis and pre-mitosis stage; and
4) M - mitosis. Here one cell actually goes through the process of division, resulting in two identical daughter cells.

Like most mammalian cells, when L-M cells are grown in monolayer they cease cycling when they become contact inhibited, and are believed to be arrested in a G_o or "G_1-like" stage (Epifanova & Terskikh, 1969). Since the replication of many viruses, including vaccinia virus, is known to be cell cycle-dependent to some degree (see Results section N), it was decided that this study should employ only cycling host cells i.e. exponentially growing suspension culture L-M cells. Confluent monolayers of L-M cells were therefore used only for purposes of virus titration.

B. VIRUS

The virus investigated exclusively in this study was the WR neurotropic strain of vaccinia virus, a member of the poxvirus group. The advantages of studying this virus include the following:

(a) It replicates exclusively in the cell cytoplasm, thereby minimizing confusion between its synthesis, and that of macromolecules in the nucleus of the host cell (but see Walen, 1971; and Oki *et al.*, 1971).

(b) It can be grown in large quantities, from either monolayer or suspension culture cells.

(c) It is easily purified (Joklik, 1962).

(d) It is stable, withstanding repeated freezing and thawing and sonication, and yet can be degraded in a controlled manner *in vitro* (Easterbrook, 1966) yielding much information as to its physical and chemical structure.

Several fairly recent review articles (McAuslan, 1969; Wilcox and Cohen, 1969; and Joklik, 1968) summarized the information available then on the poxviruses. Since that time additional information has become available. It is the purpose here to present a brief up-to-date account of the poxviruses with emphasis on the early events of replication as they relate to my studies. Some of the information contained in subsequent paragraphs can be found discussed in greater detail in an excellent current review on the reproduction of poxviruses by Moss (1974).

Poxviruses, the group of animal viruses which includes vaccinia virus, are classed morphologically as complex viruses. They are the largest animal viruses known. In view of their structural and genetic complexity, it is somewhat surprising that they have served as a favourite source of material for so many virologists' research studies. This apparent anomaly can be partly explained on the basis of the advantages listed above, and partly on the basis of the significance of poxviruses,

and especially smallpox, from a health standpoint. As recently as 1945, for example, the majority of the world's inhabitants lived in areas in which smallpox was endemic (Davis *et al.*, 1973). Local outbreaks are still occurring, and one of serious proportions was reported as recently as June 1974 in India (London Free Press, 1974). In the past intensive efforts were made to prevent and control smallpox by large-scale immunization programmes. Recently, however, North American health agency officials have decided to advise against routine smallpox vaccination of North American citizens. The real danger of rapid dissemination of the virus by today's high speed transportation of infected individuals from endemic areas makes this new policy controversial.

B1. VIRION STRUCTURE

Morphologically vaccinia virions are symmetrical, brick-shaped particles with rounded corners, as revealed by electron microscopy (Green *et al.*, 1942; Sharp *et al.*, 1946). Negative staining reveals the surface of the virion to consist of thread-like structures or ridges, 60 to 80 Å in diameter (Dales, 1963), giving the overall surface a beaded appearance. Freeze-etching (Medzon and Bauer, 1970) allows these threads to be interpreted as double ridges of parallel globular units.

The overall dimensions of the vaccinia virion are approximately 2500 X 3000 Å. Thin-sections (Dales, 1963) show a central nucleoid (Morgan *et al.*, 1954); its wall in cross section appears to consist of cylindrical pegs approximately 50 X 100 Å forming the outer layer, and an inner layer 45-50 Å thick (McAuslan, 1969). A dense dumbell-shaped core containing the DNA resides centrally in the nucleoid (Dales, 1963).

Between the nucleoid and the viral coat are two ellipsoid-shaped lateral bodies, or elliptical bodies. Using the technique of Easterbrook (1966) for controlled vaccinia virus degradation *in vitro*, it appeared that lateral bodies were attached to cores by peptide bonds. Miniely and Medzon (unpublished data) were able to remove lateral bodies by treatment with detergent and reducing agents, suggesting that the attachment might primarily be electrostatic in nature.

The chemical composition of vaccinia virus has been described in detail by Zwartouw (1964). The virion contains approximately 5% DNA, 2% lipid, 2% phospholipid and 91% protein. Trace components are usually dismissed as being contaminants of the purification procedure, but the reports (Zwartouw, 1964; Planterose *et al.*, 1962) of small amounts of RNA (maximum 0.2%) raise interesting possibilities. Very recently Roening and Holowczak

(1974) have reported the presence of significant amounts of RNA in purified vaccinia virion cores. This RNA is not covalently linked to the viral DNA genome. It specifically hybridizes with viral DNA. Its sedimentation behaviour is similar to that reported for "early" RNA sequences transcribed during the viral replication cycle. Its function is only speculative at this time, however.

The DNA has been determined to be present as one double-stranded molecule, with a sedimentation coefficient of about 65S, measuring 80-85μ in length (Easterbrook, 1967) and having a molecular weight of 160×10^6 daltons (Sarov and Becker, 1967). In theory this size of DNA can code for 400-500 proteins (Wilcox and Cohen, 1969).

Poxviruses contain 2.2% phospholipid, 2% neutral lipid and 1.3% cholesterol (Zwartouw, 1964). Presumably viral DNA does not code for any phospholipid; rather this material is probably derived from newly formed host cell phospholipid (Joklik, 1966). Using radioactive choline (a precursor of membrane lecithin) Dales and Mosbach (1968) showed that nascent phospholipid was preferentially integrated into progeny virions in infected cells. Thin-layer gas chromatography revealed that the fatty acid composition of vaccinia virus could be distinguished from that of uninfected

host cell membranes. In this way vaccinia virus can be distinguished from all other lipid-containing viruses, the lipid components of which are derived directly from pre-existing segments of cell membrane (Fenner *et al.*, 1974).

Previous approaches to determine the number of protein components in a virion have included extraction with alkaline buffers and mechanical disintegration (Marquardt *et al.*, 1965). Holowczak and Joklik (1967) treated virions with sodium dodecyl sulfate, urea and 2-mercaptoethanol, subjected the mixture to polyacrylamide gel electrophoresis, and reported the presence of at least 17 polypeptide components. Recently Sarov and Joklik (1972) reported 30 distinct polypeptide species, including 5 located at or near the virion surface, 17 in cores, and 2 glycopeptides at neither location. More recently, however, Moss *et al.*, (1973) suggested that glycoprotein might be located on the outer surface of the virion since it was removed by trypsin under conditions in which the lipids were not removed, viral DNA was not released, and no loss of plaque-forming units occurred. The genetic information necessary to code for these polypeptides represents about 20-25% of the entire vaccinia virus genome.

Reports are now accumulating which indicate

the presence of enzymes within purified vaccinia virions. All of these enzymes have been found to be associated with cores prepared *in vitro*.

The first of these enzymes to be reported was a DNA dependent RNA polymerase (Kates and McAuslan, 1967a; Munyon *et al.*, 1967). Its function is believed to be the synthesis, while vaccinia virus is at the core stage *in vivo*, (i.e. only primary uncoating has occurred) of some early mRNA using the viral DNA as template (see section B2) (iii)).

Munyon *et al.* (1968) and Gold and Dales (1968) reported a nucleotide phosphohydrolase, or nucleoside triphosphatase, in vaccinia virus. The enzyme has been shown to be dependent upon nucleic acid (Paoletti and Moss, 1972a). Moss (1974) speculates that the function of this enzyme might be the ATP-dependent extrusion of RNA from poxvirus cores (Kates and Beeson, 1970) or some kind of involvement in assembly and maturation of virus particles.

Kates and Beeson (1970) described a polyadenylate (poly A) polymerase activity in vaccinia virions. The enzyme functions in the attachment of adenylate residues to polynucleotides. The significance of the presence of poly A on the 3' termini of vaccinia

virus RNA molecules is not clear, but it is of interest to note that similar poly A tracts exist on most eucaryotic and viral mRNA species (Moss, 1974).

Pogo and Dales (1969) detected an acidic deoxyribonuclease with exonucleolytic activity, and a neutral deoxyribonuclease with endonucleolytic activity, associated with vaccinia virus cores. Pogo and Dales (1973) have suggested that the latter enzyme might function in the inhibition of host DNA synthesis. If this were the case the enzyme would somehow be required to migrate from the viral cores, in the cytoplasm, to the host cell DNA, in the nucleus. Alternatively the substrate might be required to migrate.

Tan and McAuslan (1972) reported the presence of a DNA dependent DNA polymerase associated with purified vaccinia virus, but admit that it may be a cellular contaminant.

Paoletti and Moss (1972) and Kleiman and Moss (1973) described a protein kinase activity in vaccinia virus cores. Moss (1974) suggests that the role of this enzyme is to phosphorylate the virion phosphoprotein, as described by Rosemond and Moss (1973).

Finally Faught (1974) has reported a phosphatase which may be virion associated. The enzyme

activity he described was also demonstrated in viral cores.

B2. VIRAL MULTIPLICATION

The replication cycle of vaccinia virus in host cells can be divided into a number of steps, each being characterized by unique biochemical events. For simplicity these steps are usually described as: (i) adsorption, (ii) penetration or uptake, (iii) uncoating, (iv) genome expression, (v) maturation or assembly, and (vi) release.

(i) ADSORPTION

The first stage in the vaccinia virus replication cycle is the adsorption or attachment of the virion to the host cell. Conditions affecting rates of adsorption have been investigated by Allison and Valentine (1960). Adsorption is considered to be temperature independent and cation dependent. The primary attachment is electrostatic, with the main interacting groups being amino groups of the virus and acidic groups (especially phosphate) of host cells.

(ii) PENETRATION

The method by which host cells internalize vaccinia virions is probably by a process of active ingestion akin to phagocytosis, sometimes referred to as

"viropexis" (Fazekas de St. Groth, 1948). The basis for this statement is the findings from electron microscopy, in particular the work by Dales (1963). Recently, however, a report was published which argued for fusion between the cell plasma membrane and the virus envelope as the probable mode of entry (Armstrong *et al.*, 1973). Again, the basis was electron microscopy. Dales (1973) noted a few rare cases where it appeared that fusion was indeed the method of penetration. Penetration is temperature dependent.

(iii) UNCOATING

Information on this step of vaccinia virus replication has come mainly from two lines of investigation; the electron microscopic work of Dales (1965) and the biochemical studies of Joklik (1964a, 1964b). Uncoating is divided into two stages. The first stage occurs almost immediately after penetration and results in virions being converted to cores. Coincidentally the vacuole membrane breaks down and cores appear free in the cytoplasm. This conversion, which is independent of protein synthesis, is due to the action of constitutive host cell enzymes which break down viral phospholipid and part of the protein coat. During this stage the viral DNA is not susceptible to DNAase, but serves as a message for the virion-bound DNA dependent RNA polymerase (Kates and

McAuslan, 1967a; Munyon *et al.*, 1967), the latter enzyme transcribing about 10% of the viral genome, including the message for a putative uncoating protein. The second stage results in breakdown of the nucleoprotein core by the action of the uncoating protein to liberate viral DNA which as a result becomes susceptible to DNAase. This stage requires both RNA and protein synthesis.

(iv) GENOME EXPRESSION

To simplify the description of the temporal expression of the vaccinia genome protein and mRNA molecules can be described as "early" and "late". "Early" molecules are defined as those which are synthesized prior to synthesis of progeny DNA. "Late" molecules are defined as those synthesized after progeny DNA has been synthesized. Becker and Joklik (1964) and Oda and Joklik (1967) by the technique of nucleic acid hybridization discovered that all early mRNA sequences are transcribed late but that late mRNA has sequences not found early. The "early" class of molecules can be further divided into two subclasses: molecules synthesized when vaccinia virus is at the core stage (primary uncoating) and molecules synthesized after uncoating is completed but prior to progeny DNA synthesis. By competition hybridization Kates and McAuslan (1967b) showed that mRNA synthesized at the core stage differed qualitatively from mRNA synthesized after uncoating. Thus

some control of genetic expression must exist at the level of transcription.

In addition, however, control must exist at the level of translation, for if DNA synthesis is inhibited then one finds that early proteins such as thymidine kinase (McAuslan, 1963), alkaline DNAase (McAuslan and Kates, 1966), neutral DNAase and DNA polymerase (Jungwirth and Joklik, 1965) continue to be synthesized. Apparently progeny DNA codes for a switch-off protein which inhibits translation of the stable early mRNA transcribed from parental DNA.

(v) MATURATION

Concomitantly with DNA synthesis morphological changes occur within infected cells. These changes have been visualized by electron microscopy and can be sequenced to give a logical pattern of events (Avakyan and Byckovsky, 1965). After viral DNA is released from cores, regions appear in the cytoplasm which seem to contain dense fibrous material. In these "factories" (Cairns, 1960) viral components are synthesized and assembled. First dense viral membranes appear to form around the fibrous material (Dales and Siminovitch, 1961; Dales and Mosbach, 1968). Next the dense fibrous material aggregates into clumps of filaments, the viral membranes enclose these

clumps, and "immature particles" start to form. Within the immature particle the nucleoid begins to take shape. An additional membrane encloses the condensing DNA. Finally the outer coat structures are laid down on the previously formed membrane.

(vi) RELEASE

Cells do not have to lyse in order to release progeny virions. Most virions appear to be released through cell villi by reverse phagocytosis. Exiting virions may move into the medium bathing the tissue culture cells or may be transmitted to neighbouring cells *via* villi. Nishmi and Keller (1962) showed that viral progeny in the medium amounted to only about 0.1% of the cell-bound progeny. Consequently the latter possibility seems the more likely one. In support of this statement is the observation that the presence of antivaccinia antiserum in the medium does not prevent the development of primary foci of infections.

C. EARLY CELL-VIRUS INTERACTIONS

C1. GROSS CYTOPATHIC CHANGES OBSERVABLE AT THE MACROSCOPIC LEVEL

Vaccinia virus can be considered to cause two distinct types of cytopathic effects (CPE). The first

type of CPE can be detected within 1-2 hours post-infection (PI), provided every cell is infected, and consists of cell rounding (Joklik, 1966; Hanafusa, 1962). It has been shown that an active input viral genome is not required for this kind of cell response, since heat- and ultra-violet-inactivated vaccinia virus (Hanafusa, 1962), as well as vaccinia virus in the presence of isatin-β-thiosemicarbazone (Joklik, 1966), still cause the rounding phenomenon. Thus the first type of CPE appears to be a cellular response to the ingestion of vaccinia virions for which no information encoded within the viral genome is required.

The second type of CPE begins approximately when viral progeny DNA synthesis occurs and consists of cell fusion (Appleyard *et al.*, 1962) leading to the formation of giant syncytia. This type of CPE is virus strain specific and is dependent on an active input viral genome.

The phenomenon recently reported by Ball and Medzon (1973) may be related to either of the above types of CPE. They reported that vaccinia virus-infected L-M cells increase in size within 2 hours PI and concomitantly show a sedimentation change when centrifuged in a density gradient.

C2. SYNTHESIS OF MOLECULES, OBSERVABLE AT THE MOLECULAR LEVEL, WHICH MAY ULTIMATELY LEAD TO GROSS CYTOPATHIC EFFECTS

By about 30 minutes PI Metz and Esteban (1972) were able to detect a burst of cytoplasmic RNA synthesis in vaccinia virus-infected L cells. This burst presumably represents the synthesis of viral messenger RNA due to the action of the virion-bound DNA dependent RNA polymerase (Kates and McAuslan, 1967). Later these same workers (Esteban and Metz, 1973), using high specific activity radioactive methionine, showed that vaccinia virus-specific polypeptides were synthesized as early as 20 minutes PI in L cells, having been translated from the early viral mRNA.

As early as 2 hours PI, prior to (and independent of) viral DNA replication, Ueda *et al.*, (1969) showed the presence of particulate fluorescence on the surface of vaccinia virus-infected HeLa cells using fluorescein-labelled antivaccinia rabbit globulin. The specific antigen which they detected on the cell surface did not represent stripped off outer protein layers of penetrated virions, but rather a specific protein(s) synthesized in the infected cell prior to viral genome replication. Similarly Miyamoto and Kato (1971) described the presence

of a cowpox virus-induced cell surface antigen on FL and RK1 cells, by the techniques of immune hemadsorption and fluorescent antibody without fixation. They suggested that the antigen might be newly synthesized protein translated within 3 hours PI from newly synthesized RNA transcribed from viral DNA within 1 hour PI.

Zarling and Tevethia (1971) reported that vaccinia virus-infected primary rabbit kidney cells exhibited surface changes by 2 hours PI. The change was manifested as the ability to bind the agglutinin Concanavalin A. The temporal aspect of this phenomenon, as well as its response to antimetabolites, suggested that it might be related or equivalent to the phenomena described by Ueda *et al.* and by Miyamoto and Kato.

Harry and Medzon (1974) recently reported the ability to detect a vaccinia virus specific antigen on the surface of L-M cells by 45 minutes PI, by treating infected cells with ^{125}I-labelled IgG specific for vaccinia virus. This antigen showed close similarity to virus surface antigen. Its synthesis was dependent on RNA and protein synthesis, but independent of viral DNA replication.

D. SEDIMENTATION DIFFERENCES OF CULTURED MAMMALIAN CELLS AND METHODS FOR DETECTION OF SUCH DIFFERENCES

D1. NATURE AND BASIS OF THE SEDIMENTATION DIFFERENCES

A population of mammalian cells in suspension can be subjected to separation procedures which take advantage of physical differences, exhibited by individual members of the cell population, in parameters such as cell volume (often referred to as cell size), cell density, net surface charge, and solubility in two immiscible liquids (counter-current distribution or phase partition). A cell population which can be separated based on one or more of the parameters listed above may consist of a mixture of different cell types (e.g. blood cells consisting of platelets, erythrocytes, lymphocytes, monocytes and granulocytes) or it may consist of quite homogeneous members (e.g. a cell line culture or cell strain culture). In the former case one might expect major differences to exist in the members of the cell population, but in the latter case one might predict no differences in the physical properties of the members of the population. This prediction has been shown in many cases to be false, however, by the use of rather sensitive techniques which can detect subtle differences, for example

sedimentation properties, in members of a population.

The two types of separation which have been used extensively in separating populations of mammalian cells are sedimentation-rate separation and buoyant-density separation (Shortman, 1972).

Basis of sedimentation-rate separation

If mammalian cells in suspension are considered to be spherical particles, then when they move through a uniform stable medium under the influence of a constant gravitational or centrifugal force they rapidly reach a constant velocity, the value of which depends on both the density and size of the cell (Miller and Phillips, 1969). However, for mammalian cells it has been shown (Miller and Phillips, 1969; Shortman, 1972) that this velocity depends primarily on the radius of the cell i.e. sedimentation velocity is dependent primarily on cell size.

When a cell is subjected to a centrifugal field (a gravitational force greater than unit gravity), the sedimentation velocity at a given time and given position in the gradient becomes a function of cell size, angular velocity, density and viscosity of the medium, and density of the cell (Shortman, 1972; Boone, Harell and Bond, 1968).

Boone *et al.* (1968) devised a computer program to give the sedimentation position and velocity of cells being centrifuged in a specified rotor under defined conditions.

Basis of buoyant-density separation

Theoretically it should be possible to separate mammalian cells based on differences in their buoyant densities. This has been shown to be the case with bacteria (Hildebrand and Pollard, 1969). Nias and Fox (1971) argue that the method is unsuitable for mammalian cells because such cells show practically no buoyant density differences (Anderson, Peterson and Tobey, 1970) despite volume variations up to two-fold (Terasima and Tolmach, 1963). Shortman (1972) admits that the density range of mammalian cells is narrow, that separation therefore depends on small differences in cell composition, but feels that such separation can be applied to mammalian cells. As with the separation of macromolecules the underlying principle in this form of separation is that cells centrifuged in a density gradient will rise or sink until they reach that region of the gradient where the density is equal to their own. Additional centrifugation, after isopycnic banding has occurred, will not result in a repositioning of the cells. Unlike sedimentation-rate

separation which depends on the size, shape, and density of cells, buoyant (equilibrium)-density separation depends solely on cell density (Shortman, 1969).

D2. TECHNIQUES COMMONLY USED TO DETECT SEDIMENTATION DIFFERENCES

(a) Sedimentation-rate separation

The first stable system for sedimentation-rate separation of mammalian cells was described by Mel (1964). The procedure, which was given the acronym "staflo" for stable-flow free boundary, gave some fractionation of bone marrow cells under unit gravity conditions. For technical reasons the "staflo" system cannot resolve small cells of similar size (Shortman, 1972).

Miller and Phillips (1969) devised a simpler apparatus than that used by Mel, based on the same principle of zonal sedimentation velocity separation at unit gravity. Their system, which was given the name "staput", was shown to be able to separate a population of sheep erythrocytes on the basis of cell size. The theoretically calculated and experimentally measured sedimentation velocities for these cells agreed very well.

Mitchison and Vincent (1965) developed a method for separating a suspension culture of exponentially growing yeast cells by gently centrifuging them through a sucrose gradient. The principle of the separation was differential sedimentation, and the property by which the cells differed from each other was size (volume). It is known that as cells traverse the eucaryotic cell cycle (G1,S, G2, M) they double in volume. This volume doubling can be shown to result in an increase in the velocity of sedimentation by a factor of 1.59 (Miller and Phillips, 1969). Therefore cycling cells should be separable by sedimentation-rate separation.

Three weeks after Michison and Vincent published their report Sinclair and Bishop (1965) described the adaptation of the method to mammalian cells, in particular to exponentially growing suspension culture strain-L mouse cells. The first report which took advantage of these results to obtain a sub-population of an exponentially growing culture, homogeneous with respect to the stage of the cells in their traverse around the cell cycle, was that of Morris *et al.* (1967). Using a neoplastic murine mast cell culture they were able to obtain a sub-population of cells which were active in synthesizing DNA (S phase). Later Ayad *et al.* (1969), using gradients made from the high molecular

weight polymer Ficoll, reported the separation of an asynchronous population of mouse lymphoma cells into three synchronous sub-populations (G2, S and G1).

Ayad's technique was exploited with considerable success by Creighton (1970) who was able to separate L-M cells on a continuous Ficoll gradient into fractions based on the cell cycle. She showed that G1 cells possessed 2 to 8 times more carbohydrate per unit protein than older cells in other phases of the cell cycle. Furthermore G1 cells had greater enzymatic activity per unit of surface area or per unit volume, and were most affected in their subsequent growth by the addition of sera. Her conclusion was that the L-M cell membrane varied chemically, enzymatically and immunologically as the cell progressed from G1 through the cycle to M.

Lippman (1970) presented the only argument to date refuting sedimentation rate as the basis for separating a homogeneous population of mammalian cells (which she termed a "primary population"). She presented data showing that the density of an L-M suspension culture cell does not remain constant throughout the cell cycle, that consequently variations in cell density are not insignificant, and that as a result the sedimentation velocity is dependent on cell density as well as

cell size. Lippman and Mathews (1970) devised a two-step combination technique, which they termed "rate-density sedimentation". According to their protocol L-M suspension culture cells are subjected to an initial velocity sorting process by centrifugation in low concentrations of Ficoll at the top of a centrifuge tube with density gradient below. The rate sorted cells which result are then introduced into their isopycnic positions by controlled increments in centrifugal velocity. Cells, homogeneous with respect to volume, rate sedimented according to density. The technique sorted the population into subpopulations unique in volume and density. Finally it was shown that the density variation was a function of the cell cycle. Lippman argued that Ayad *et al.* and Fox and Pardee (1970) made the mistake of using a density range for their gradients which was precisely that of the isopycnic density range of the cells which they used.

A modification of the "staflo" and "staput" systems was described by Boone, Harell and Bond (1968). They were able to separate artificially mixed populations of cells with great purity by using the principle of zonal centrifugation. The principle is the same as for the systems mentioned above, but now the cells are centrifuged through a viscous stabilizing gradient. Under a set of standard conditions, defined by the authors and

consisting among other things of a gradient of 10-20% w/w Ficoll in SMEM medium, Boone *et al.* (1968) used a computer program based on a differential sedimentation equation to determine sedimentation distance and sedimentation velocity versus time for any cell with a known diameter and density. Pretlow and Boone (1969) later experimentally verified these computer predictions. Still later MacDonald and Miller (1970) slightly modified the "staput" apparatus and showed that a population of asynchronously dividing L cells could be separated primarily on the basis of cell size.

Another type of sedimentation velocity separation is centrifugal elutriation (Lindahl, 1948). Elutriation is a method of separating particles according to their rates of sedimentation in which a liquid (or gas) containing the suspended particles is flowed upwards against gravity. The principle of separation is that particles with sedimentation rates less than the upward rate of flow of the liquid are washed away from the larger heavier particles. By using a centrifuge particles which would sediment only slowly at unit gravity can be treated as well. McEwen *et al.* (1968) used this principle to separate lymphocytes and granulocytes from leukocyte-enriched blood. The major drawbacks of centrifugal elutriation are the necessities of using either special

centrifuges or special rotors.

Albertsson (1972) described a new method for separating cells based on sedimentation at unit gravity. The method was coined "multiple sedimentation" (Walter and Albertsson, 1971). The underlying principle was that the separation obtained by a single sedimentation could be increased by a multi-stage procedure analogous to counter-current distribution between two immiscible phases. The basis of the separation was cell size.

(b) Buoyant-density separation

There are three types of buoyant density gradients which can be used for buoyant-density (iso-pycnic) separation.

One type is the continuous gradient. The first report of effective separation of mammalian cells on continuous gradients was made by Leif and Vinograd (1964) who separated human erythrocytes on bovine serum albumin gradients based on differences in their buoyant densities. Later Leif technically improved the system. Pertoft (1969) has reviewed the literature on the use of gradients of colloidal silica to separate blood cells, liver cells, bone marrow cells and other cell types.

A second type is the discontinuous gradient.

This type of gradient is commonly used because it is quite simple to construct, unlike the continuous gradient which requires special care to construct accurately and precisely. Discontinuous gradients have limitations in their capacity to separate cells on the basis of differences in density (Shortman, 1972) since the only operative part of the gradient is the zone at each interface.

The third and last type of density gradient is the neutral-density or uni-density gradient. The centrifugation of such gradients is sometimes referred to as "isopycnic centrifugation" (Cutts, 1970) (in contradistinction to "isopycnic gradient centrifugation" of continuous or discontinuous gradients). The gradient, consisting of material at only one density, can separate from a population of cells those members which have a density equal to that of the gradient material. Cells having a lighter density remain above the gradient and those with a greater density pellet at the bottom of the gradient. Moore *et al.* (1972) used the uni-density gradient (which they termed a "density cut") to partially separate colony forming cells from a majority of nucleated marrow cells. Shortman *et al.* (1972) separated damaged cells (which pelleted) from mouse spleen cell suspensions, leaving erythrocytes and intact nucleated cells in the supernatant fraction.

E. SEDIMENTATION CHANGES INDUCED IN CULTURED MAMMALIAN CELLS BY VIRUS INFECTION

Over the past four years 6 reports have appeared in the literature describing changes in the sedimentation properties of mammalian cells in culture as a result of being infected with viruses.

The first of these reports was made by Sykes *et al.* (1970). Believing that virus-infected cells might have a greater buoyant density and behave differently than virus-free cells when centrifuged on a discontinuous gradient, Sykes *et al.* examined the sedimentation distribution pattern of vaccinia virus-infected CMP and ME-180 cells, and poliovirus-, influenza virus-, and echovirus-infected ME-180 cells. Detailed results were reported only for vaccinia virus-infected cells. 48 hours PI (4-5 days prior to development of gross CPE with the quantity of virus used) a two-fold increase in the percentage of cells in the topmost zone of a Ficoll gradient compared to controls was noted. Furthermore cultures derived from the upper zones showed massive CPE 4 days after culture, whereas cultures from the lowest zone were apparently normal when discarded. Thus a definite change was noted in the sedimentation distribution pattern of vaccinia virus-infected cells, although the direction of the change was contrary to that

expected (infected cells exhibited an apparent decrease in buoyant density rather than the anticipated increase).

Simian virus 40 (SV40) is known to induce some cells in a confluent monolayer to replicate DNA and divide. Arguing that those cells which are induced to synthesize cellular DNA by SV40 (S phase) should be larger in size than non-induced cells (G1 phase), Fox and Levine (1971) subjected SV40-infected confluent 3T3 cells to Ficoll density gradient centrifugation. They found that indeed virus-induced cells were partially separated (sedimented further) from non-induced cells, 24 hours PI. They were also able to show a link between SV40 induction and transformation by this technique; that is induced cells exhibited 3 to 8-fold higher transformation frequencies than non-induced cells.

Sugawara *et al.* (1971) reported that Epstein-Barr virus-carrying Burkitt lymphoma cells could be separated from uninfected cells on a discontinuous gum acacia gradient after 6 days of incubation. Virus-carrying cells were detected by immunofluorescence. Although the general cell population contained only 5-20% virus-carrying cells, they were able to obtain a zone of cells, at the top of the gradient, in which 70-100% of the cells were positive in intracellular and membrane immunofluorescence tests. Two days later (after 8 days incubation)

more cells were found in the top zone of the gradient and more of them were positive.

By 2 hours PI Ball and Medzon (1973) noted that vaccinia virus-infected L-M cells exhibited an altered sedimentation distribution pattern in a discontinuous Ficoll gradient. The alteration consisted of a shift of cells towards the top of the gradient and was due to an increase in cell size (and thereby a possible decrease in buoyant density). Cells which shifted toward the top contained 4 times more infected members than cells which remained nearer the bottom of the gradient. The shift was both time dependent and multiplicity of infection (MOI) dependent.

Another report which described a volume change of virus-infected cells was that by Naeve *et al.* (1974). The authors did not physically separate virus-infected cells from uninfected cells, however. They simply showed that Venezuelan equine encephalitis virus could induce volume changes in mosquito cells in culture and that such changes could be detected without visible morphological evidence of viral infection. Detection was accomplished by the use of a Coulter spectrometer and computer program, which together gave a volume distribution profile of cells 2 days PI.

Very recently Ross and Ash (1974) described a density change in Herpes simplex virus-infected L-M, chick embryo fibroblast, and HEp-2 cells, by 3 hours PI (2 hours beyond the adsorption period, and 2-3 hours prior to appearance of progeny virus). The method of detection of this change in density was centrifugation on Ficoll gradients. Immediately after the adsorption period cells exhibited a transient increase in density, but with time of incubation after the adsorption period this trend was reversed such that the cell density continually decreased beyond the 0 hour PI value. The authors argue for the necessity of cellular, but not viral, protein synthesis for the occurrence of the cell density decrease.

MATERIALS AND METHODS

(1) CELL

(a) GENERAL MANIPULATIONS

The cell strain used exclusively in this study was L-M (American Type Culture Collection CCL 1.2), purchased from the American Type Culture Collection (Rockville, Maryland). This strain was derived from NCTC clone 929 which in turn was derived from the parental strain L. Strain L cell was derived from a normal 100 day-old male mouse in 1940. In 1962 L-M cells in their 113th serial subculture in protein-free medium were submitted to the Animal Cell Culture Collection by D. J. Merchant.

L-M cells are morphologically considered to be fibroblast-like, when growing in monolayer on a glass or plastic surface. They exhibit "contact inhibition" of growth when confluency is reached. These cells can also be grown in monodisperse suspension culture. In this case the morphology of the cell changes from fibroblast-like to spherical.

The L-M cells used in this study were tested routinely for the presence of contaminating mycoplasma/

PPLO and at all times were found to be contaminant-free. The cells were also subjected to electron microscopic examination for the presence of endogenous mouse viruses. They were found to be free of virus and C-type particles (Dr. Janet Hartley, personal communication).

Suspension cultures were prepared from monolayers by gently scraping the cells off the glass or plastic surface with a rubber policeman. After brief trituration to break up large clumps, the concentration of the cell suspension was adjusted with medium and the suspension transferred to a screw-capped siliconized Erlenmeyer shaker flask. The flask was incubated at 37°C on a gyrotory shaker (New Brunswick Scientific Company, New Brunswick, New Jersey) at 100-110 rpm. The volume of a suspension culture was never allowed to exceed 40% of the total volume of the flask in which it was incubated, to obtain adequate agitation and oxygenation.

Monolayers were prepared from suspension cultures by adjusting the concentration of the suspension with medium and introducing the culture into either screw-capped glass prescription bottles, which were then tightly sealed and incubated in the horizontal position at 37°C, or plastic Multi-dish Disposo Trays (Linbro Chemical Company, New Haven, Conn.) which were incubated in an atmosphere of humidified 95% air-5% CO_2 at 37°C. Roller

bottle monolayers were prepared as outlined in section 7(b).

(b) CELL COUNTING

The concentration of a suspension culture of cells was routinely determined on a model A Coulter Counter (Coulter Electronics, Kenmore, Chicago) using a 100 μ diameter orifice. Threshold settings were periodically checked against a suspension of ragweed pollen. A given sample was counted at least twice and the values averaged. Samples for counting were obtained by mixing 0.5 ml of a suspension culture of L-M cells with 24.5 ml isotonic saline.

(c) CELL VIABILITY DETERMINATIONS

The viability of a suspension culture of cells was routinely determined by the vital stain dye exclusion test. In this case 0.5 ml of a suspension culture was mixed with 0.1 ml of a saturated solution of erythrosin B in phosphate buffered saline and left undisturbed for 5 minutes. After this a drop of the suspension was placed on a glass microscope slide, a coverslip gently lowered on top and an estimate made by phase contrast microscopic observation at 100 X magnification, of the percent cells excluding the dye.

(d) CELL SIZING

Due to small numbers of cells in any one fraction from a Ficoll gradient, it was technically difficult to perform a size analysis on cells from a given fraction. Consequently entire unfractionated cell populations were analyzed. Using an MOI of 10, cell cultures were analyzed using two different apparatuses. Early in this study use was made of a Cytograf (Bio/Physics Systems Inc., Mahopac, New York). With this instrument cells were sized by passing them in single file fashion through a channel which was intersected at right angles by a focused laser beam. Size results were displayed on an oscilloscope in the form of a histogram. Comparison of these histograms gave a qualitative idea of cell sizes. Later in the study samples were analyzed on a Celloscope counter (Particle Data Inc., Elmhurst, Illinois) linked to a Nuclear Data pulse height channel analyzer and chart recorder. Since the data from the counter provided only relative size values, standard particles of known diameter (mulberry pollen, 12-13 μ, and ragweed pollen, 19-20 μ) were also analyzed to estimate changes in absolute cell diameter.

The principles upon which these apparatuses are based can be found outlined in Appendixes 5 and 6.

(2) MEDIUM

The medium used throughout this study was serum-free medium 199 (Morgan, Morton and Parker, 1950). A batch of 20 liters was routinely made by first dissolving 220 gm dried powder (Difco Laboratories, Detroit, Michigan) in 18 liters distilled deionized water. The resultant "199-water" was filtered under positive pressure (approximately 20 psi nitrogen) through a 0.22 μ pore 142 mm diameter filter (Microfil, RB Filters Ltd., Toronto, Ontario) into 400 ml screw-capped prescription bottles already containing 35 ml 10X autoclaved peptone-water or peptone-methylcellulose water (see below). Medium bottles were aseptically filled to 350 ml, capped tightly, dated, and stored at 4°C.

In this study the following compounds were routinely added to 199-water during preparation of a batch:

For monolayer cultures 0.5% w/v Bacto-peptone (Difco Laboratories, Detroit, Michigan) was added (Medzon and Merchant, 1971). This was accomplished by dissolving 100 gm peptone in 2 liters of water, dispensing 35 ml of the resultant 10X "peptone-water" into each empty medium bottle, and autoclaving the bottles at 120°C for 20 minutes. 315 ml filtered 199-water (see above) was aseptically added to the sterile peptone-water. This gave medium 199P.

For suspension cultures 0.5% w/v peptone plus 0.12% w/v methylcellulose (Methocel, 15 cps grade, Dow Chemical Co., Midland, Michigan) were added (Merchant *et al.*, 1964). This was accomplished by dissolving 100 gm peptone plus 24 gm methylcellulose in 2 liters of water, dispensing 35 ml of the resultant 10X "peptone-methylcellulose-water" into each empty medium bottle, and autoclaving as above. Aseptic addition of 315 filtered 199-water to each bottle gave medium 199P+MC.

Each batch of medium was sterility tested by randomly selecting bottles for incubation. Prior to incubation 4-5 ml of 5% w/v sterile sodium bicarbonate was added to each bottle to adjust the final pH to approximately 7.2. Bottles were incubated at 37°C for 48-72 hours and observed for growth of contaminants.

Prior to use every bottle of medium 199P or medium 199P+MC received 3.5 ml each of sterile 10X L-glutamine, and 10X penicillin+ streptomycin, such that the final concentrations of these additives were: 100 µgm/ml, 100 units/ml and 100 µgm/ml respectively. The glutamine and antibiotic concentrates were prepared by dissolution in distilled, deionized water followed by filtration sterilization. The sterile solutions were stored frozen at -20°C in 3.5 ml aliquots and thawed for

use. The final addition to each bottle of medium was 4-5 ml of 5% w/v autoclaved sodium bicarbonate, to raise the pH to approximately 7.2.

(3) VIRUS

(a) GROWTH OF STOCK VIRUS

The strain of vaccinia virus used throughout this study was the WR (Western Reserve) strain, neurotropic for the mouse, which was originally obtained from Dr. J. E. Officer, Fort Detrick, Maryland. Stock virus was stored frozen at -90°C.

Vaccinia virus was routinely grown in exponential phase suspension cultures of L-M cells. Such cells were first centrifuged for 15 minutes at 1000 x g. Medium 199P+MC was removed and the cell pellet was resuspended in thawed, sonically treated stock vaccinia virus plus fresh medium 199P+MC to give a final cell concentration of approximately $5x10^7$ cells/ml. This material was slowly mixed at 35°C for one hour for virus adsorption, after which fresh medium 199P+MC was added to achieve a final cell concentration of about $5x10^5$ cells/ml. The infected cell suspension was then incubated for 48-72 hours at 35°C in a screw-capped Erlenmeyer flask in a gyrotory shaker incubator.

After 48-72 hours the infected cells were centrifuged for 15 minutes at 1000 x g. The supernatant was discarded and the cell pellet was resuspended in a small volume of Tris-EDTA buffer (10^{-3}M) and sonicated, while cooled in ice water, for several minutes with an MSE ultra-sonic probe (Measuring and Scientific Equipment Ltd., London, England) (Medzon and Bauer, 1970). This material was centrifuged for 15 minutes at 1000 x g to sediment large cellular debris. The supernate, containing the virus, was layered over 36% sucrose w/v in Tris-EDTA buffer and centrifuged in a fixed angle rotor (Sorvall SS34) or swing-out head (Sorvall HB4) for 30 minutes at 39000 x g or 60 minutes at 25000 x g, respectively. The virus pellet was then resuspended in 10^{-3}M Tris, pH 9.0 and stored at -90°C. All of the above steps were carried out aseptically.

(b) VACCINIA VIRUS TITRATION

Titrations in this study were accomplished entirely by the plaque assay method using confluent monolayers of L-M cells. Suspension culture cells were first diluted with medium 199P to achieve a cell concentration of approximately 4-5 X 10^5 cells/ml. 3.0 ml of this suspension were placed in each depression or

"well" of a Multidish Disposo Tray (Linbro Chemical Co., Inc., New Haven, Conn.) and the trays were incubated at 37°C in a humidified atmosphere of 95% air-5% CO_2. By 16-20 hours the cells were usually confluent. After removing the medium over the monolayers by aspiration, 1.0 ml aliquots of 10-fold dilutions of the sonicated virus in medium 199P were added to each well. The trays were replaced in the 95% air-5% CO_2 atmosphere and rocked slowly for one hour for effective adsorption. After this time an additional 2.0 ml of fresh medium 199P were added to each well, the trays returned to their humidified air-CO_2 environment, and left undisturbed at 37°C for 48-72 hours.

After 48-72 hours the trays were removed from the incubator and processed for plaque quantitation. First the 3 ml medium in each well were aspirated off. Then the cells of the monolayer were fixed by covering the monolayer with neutral buffered formalin (Merchant *et al.*, 1964) followed by staining with filtered 0.1% w/v crystal violet solution. Finally plaques were counted using a Jena dissecting binocular microscope and underlying grid. Plaque counts between 30 and 300 per well were considered to be statistically significant. Each virus dilution was titrated in triplicate and the results averaged.

(c) PREPARATION OF RADIOACTIVE VACCINIA VIRUS

The procedure used to prepare radioactive vaccinia virus, labelled in the DNA with ^{3}H-TdR, was that of Sarov and Joklik (1972), with some modifications. Briefly, 2 X 10^{8} log phase L-M suspension culture cells were infected with 2 X 10^{8} pfu of stock vaccinia virus, following the method outlined in 3(a) above. After adsorbing the virus to the cells for 40 minutes at 4°C, fresh medium 199P+MC was added to reduce the cell concentration to approximately 5 X 10^{5} cells/ml. These cells were then placed in a shaker flask and incubated at 37°C in the gyrotory shaker. After 1 hour of incubation 200 µCi. of ^{3}H-TdR was added to the suspension (i.e. 1 µCi/10^{6} cells), and incubation was continued for a total of 48 hours.

The virus prepared in this manner was purified according to the method of Joklik (1962). Sucrose was removed by centrifugation and replaced with either Tris buffer or medium 199P+MC. Virus prepared in this manner was found to have a specific activity of 6.7 X 10^{-6} cpm/pfu, and a titer of 10^{10} pfu/ml.

In addition (and subsequent) to preparing radioactive virus as just described, some higher specific activity ^{3}H-TdR-labelled vaccinia virus was kindly

supplied by Dr. W. K. Joklik. It was found to have a specific activity of 2.8×10^{-4} cpm/pfu and a titer of 1.2×10^{8} pfu/ml.

(4) FICOLL DENSITY GRADIENTS

(a) FICOLL SOLUTIONS

Gradients were made from Ficoll (Pharmacia, Uppsala, Sweden), a high molecular weight polymer of sucrose (average molecular weight= 400,000 daltons). Ficoll solutions were made in either of the following ways:

(i) For routine nonsterile work a stock solution of Ficoll was prepared at a concentration of 18% (w/v) by dissolving Ficoll powder in medium 199P at $4^{\circ}C$ with vigorous stirring. After dissolution the pH of the medium was adjusted to 7.2 with sodium bicarbonate solution. The stock solution of Ficoll was stored frozen between uses at $-20^{\circ}C$. Exceptionally (for determinations of maximum concentrations of gradients) solutions greater than 18% were used.

(ii) For sterile work 18 gm of Ficoll was autoclaved for 15 minutes at approximately $112^{\circ}C$ (reduced steam pressure) and then dissolved in sterile medium 199P plus antibiotics

and bicarbonate to a final volume of 100 ml, as above. This material was also stored frozen between uses.

(b) DENSITY GRADIENTS

(i) Small gradients

Each gradient consisted of five layers, each 2 ml in volume. The concentration of Ficoll in the layers were 14, 15, 16, 17 and 18% (w/v) in medium 199P. As each layer was added it was allowed to slowly run down the inside wall of a 16- by 100-mm siliconized glass culture tube, thereby forming clearly visible interfaces. All Ficoll solutions used were kept at 4°C during gradient preparation.

(ii) Large gradients

Each gradient consisted of five layers, each 6 ml in volume. The various Ficoll concentrations were as above. The tube used was a 28- by 104-mm polycarbonate Sorvall centrifuge tube.

(c) CENTRIFUGATION OF CELLS THROUGH GRADIENTS

(i) Small gradients

4 to 5 ml of infected and mock-infected cell suspensions, at concentrations of between 2 and 5 X 10^5

cells/ml, were removed from 37°C incubation at the appropriate time post infection and chilled briefly on ice. After chilling 2.5 ml of each culture were gently layered on top of a small gradient. Gradients were centrifuged in a Sorvall superspeed RC2-B centrifuge (Ivan Sorvall, Inc., Newtown, Conn.) using a Sorvall HB4 swinging bucket rotor, at 1000 x g for 15 minutes at 4°C under manually controlled gradual acceleration conditions. Deceleration was not manually controlled. After centrifugation the interfaces were still clearly visible. Centrifuged cells formed opalescent bands at these interfaces. Gradients were unloaded from the top either automatically by a Buchler Densiflow density gradient remover (using 70% sucrose to displace the gradient) or manually with a Selectapette (Clay Adams, Parsippany, New Jersey).

(ii) Large gradients

10 to 15 ml of cell suspensions were removed from 37°C incubation and chilled briefly on ice. After chilling 8.6 ml of each culture were gently layered on top of a large gradient. Gradients were centrifuged in a model PR2 International portable refrigerated centrifuge (International Equipment Co., Needham Heights, Mass.) using swinging bucket rotor #269 at 1000 x g for 15 minutes at 4°C under acceleration and deceleration conditions as noted

above. Gradients were unloaded manually from the top with a 5 ml glass pipette linked to a 5 ml glass Cornwall syringe via a "pipet adapter" (Fisher Scientific Co., Pittsburgh, Penn.).

(5) INFECTIOUS CENTER ASSAY

2.5 ml aliquots of infected suspension cultures were centrifuged on small sterile 14-18% discontinuous Ficoll gradients. To facilitate aseptic fractionation manual fractionation with a Selectapette was carried out. 1.0 ml fraction volumes were taken. A sample from each fraction was serially diluted through a series of 10-fold dilutions, and 1.0 ml samples of each dilution were placed onto duplicate or triplicate preformed confluent L-M cell monolayers in Multidish Disposo Trays. 2.0 ml of medium 199P were added to each well and the trays were incubated in the standard way. After 48-72 hours cells were fixed and stained, and the number of infectious centers/ml determined.

(6) INCORPORATION OF RADIOACTIVE PRECURSORS

(a) TRITIATED THYMIDINE (^{3}H-TdR)

To measure DNA synthesis LM cells in suspension were routinely treated in the following manner: 0.9 ml aliquots were removed from the culture flask and placed

in 7.5 ml siliconized bijoux bottles. To each bijou bottle was added 0.1 ml of a 10X concentrate of ^{3}H-TdR in medium 199P+MC (10 µCi/ml; specific activity 28 mCi/mgm) (New England Nuclear, Montreal, Quebec). The bijou was capped tightly and rotated on a 6 rpm rotissary in a 37°C incubator. After 1 hour incubation the bottle was removed from the incubator, chilled on ice, and immediately thereafter three 0.1 ml aliquots were removed and centrifuged on a clinical bench-top centrifuge to pellet the cells. Supernatants were discarded and pellets were resuspended in approximately 1.0 ml of room temperature 0.25 N NaOH (to lyse the cells) according to the method of O'Shaughnessy *et al.* (1972). After 10 minutes 9-10 ml of ice-cold 25% trichloroacetic acid (TCA) was added, the material mixed well, and filtered by suction through 0.22 µ or 0.45 µ pore 25 mm diameter RB filters. Each tube was rinsed twice with cold 5% TCA and the washings were filtered as well. Filters were then dried under a heat lamp and placed in plastic scintillation vials (New England Nuclear) containing 10 ml of a cocktail consisting of 0.4% (w/v) 2,5-diphenyloxazole + 0.01% (w/v) 1,4-bis [2 - (4-methyl-5-phenyloxazolyl)] benzene in toluene, all of these ingredients being of scintillation grade. Vials were then counted in a Philips Liquid Scintillation Analyzer (PW4510, Philips Electronics Industries, Ltd., Toronto, Ontario), operating with an

efficiency for tritium of approximately 54%.

(b) TRITIATED URIDINE (^{3}H-UR)

To measure RNA synthesis cells were treated as in (a) above but with a 10X concentrate of ^{3}H-TdR replaced by 10X ^{3}H-[redacted] in medium 199P+MC (100 µCi/ml; specific acitivity 106 mCi/mgm) (New England Nuclear).

(c) TRITIATED AMINO ACID MIXTURE

To measure protein synthesis cells were treated as in (a) above but with a 10X concentrate of ^{3}H-TdR being replaced by 10X ^{3}H-L-amino acid mixture in medium 199P+MC (10 µCi/ml) consisting of a combination of 15 purified L-amino acids.

In all cases (a-c above) the triplicate samples were counted in the scintillation counter for a minimum of one minute. Results were always averaged and expressed as counts per minute. Background values (usually about 15 cpm) were always subtracted prior to plotting results.

(7) PREPARATION OF M PHASE CELLS

Mitotic phase cells were prepared by two different methods: induction and selection.

(a) INDUCTION

Colchicine, used for many years in research on plant genetics (for doubling chromosomes), has more recently been applied to studies of mammalian cells in tissue culture. In particular the derivative colcemid (demecolcine) is capable of reversibly arresting cells at the metaphase-anaphase region of the cell cycle (Puck and Steffen, 1963). The action of these drugs is believed to be at the level of assembly or function of the mitotic spindle. For reversibility of action the concentration of colcemid must be kept low (Stubblefield, 1964) and/or it must be present in the medium for only a short period of time. If reversibility is of no concern, however, longer exposure and/or higher concentrations of the drug are permissible.

Log phase suspension cultures were used as the source for M phase cells. An appropriate volume of stock colcemid at 1 mgm/ml (Ciba Pharmaceuticals, Don Mills, Ontario), kindly supplied by Dr. C. F. Robinow, was introduced into the suspension flask so that the final concentration of colcemid in the medium was 2 µgm/ml. This concentration was 20 times greater than that recommended by Robbins (1969) for treating monolayer cultures. Cells were monitored periodically thereafter

by phase contrast light microscopy for mitotic index and by Coulter counting for cell concentration.

(b) SELECTION

Based on the procedure of Terasima and Tolmach (1963), which makes use of the property that monolayer cells often round up and adhere much less firmly to the surface during mitosis, Sanders, Medzon and Ball (unpublished results) devised a simple and inexpensive roller bottle apparatus to maximize yields of M phase cells from L-M cell monolayers. Figure 23 shows the back and side view of the apparatus.

To obtain near-confluent monolayers overnight each 26 x 11 cm screw-capped glass roller bottle (Johns Scientific, Toronto, Ontario) was filled with 200 ml of a suspension culture of L-M cells, at a concentration of approximately 2×10^5 cells/ml. The source of such cells was an exponential phase suspension culture, which was diluted appropriately with medium 199P. No gassing of the roller bottle was required. The bottles were rotated on the apparatus at 1 rpm in a 37°C incubator.

Mitotic cells were collected by the method of Brent (personal communication). First the inoculum medium was poured off and replaced with 100 ml warmed medium

199P. The cell sheet was washed with the fresh medium by swirling the medium around the inside of the bottle. The wash medium was poured off and the washing procedure repeated once. Finally 100 ml warmed fresh medium 199P+MC was introduced into the bottle and the bottle rolled for 60 minutes. The 60-minute-wide "window" of cells was harvested by vigorously swirling the medium over the monolayer for a total of about 1 minute. The medium was decanted, centrifuged for 5 minutes at 4°C to pellet the cells, and the cells resuspended in cold medium 199P+MC and placed in a culture tube in ice. Cells were immediately processed for mitotic index determination and counted on a Coulter counter to determine the number of cells retrieved and thereby calculate the percent yield.

(c) PROCESSING OF CELLS FOR MITOTIC INDEX DETERMINATION

(i) Method of Schindler *et al.*

In the early part of this study cells were treated, with minor modifications, according to the method of Schindler *et al.*(1967) to determine mitotic indices. Cells were fixed by mixing an aliquot of the suspension culture with an equal volume of fresh ethanol-acetic acid-water (5:2:3, v/v). Cells were then centrifuged, the fixative drained off well, and the cells resuspended in a small volume of 0.05% crystal violet in 1% acetic acid.

A drop of this suspension was placed on a microscope slide and covered with a coverslip. Examination was performed on a Zeiss binocular phase contrast microscope at a magnification of 500 X. Only cells exhibiting distinct chromosomes were scored as mitotic phase cells.

(ii) Method of Brent

For the latter part of this study the method of Schindler *et al.* was replaced by that of Brent. According to this method (Brent, personal communication) approximately 10 ml ice cold phosphate buffered saline were added to the cell suspension. The cells were then centrifuged and resuspended in 5 ml 0.1 M ice cold sucrose with constant agitation. This step caused the cells to swell due to hypotonicity. After standing on ice for 3 minutes the cells were centrifuged and resuspended in 5 ml fresh methanol-acetic acid (3:1 v/v) with constant agitation. After standing on ice for 10 minutes the cells were centrifuged and finally resuspended in 1-2 drops of the fixative. A drop of this material was applied to a microscope slide and air dried. Examination was performed as above. This method caused far less cell distortion and more clearly delineated the chromosomes than method (i). Quantitatively the two methods were equivalent. Method (ii) was the method of choice when autoradiography plus

mitotic scoring was required, in view of the minimal distortion of the chromosomes by this method and the need to determine clearly if silver grains overlay chromosomes.

(8) AUTORADIOGRAPHY

After pulsing cells with 5 μCi/ml ^{3}H-TdR for 1 hour at 37°C (to detect S phase cells) they were washed several times to remove unincorporated radioactivity. After resuspension a drop of cell suspension was placed on a clean glass microscope slide and air dried. The slide was fixed in fresh methanol-acetic acid (3:1 v/v) for 10-30 minutes. After rinsing off the fixative the slide was placed in cold 5% TCA for 10 minutes. It was then rinsed well and placed in 70% ethanol for 60 minutes. Then it was air dried.

At this point slides were ready to be coated with emulsion. The emulsion used was Kodak NTB2. It was prewarmed in a water bath in a darkroom at 42-45°C for 1 hour. Then slides were dipped once slowly into and out of the emulsion, and excess emulsion was drained and finally wiped off with a paper towel. The slides were left to dry for 2 hours. After the drying period the slides were placed in a light-tight box containing a dessicant. The box was sealed well with black tape and transferred from the darkroom to a refrigerator at 4°C.

After 6-7 days of exposure slides were ready to be developed.

Developing consisted of placing the slides in the following solutions for the following lengths of time: 1) Developer - Kodak D-76 1 1/2 minutes

or D-19 6 minutes

2) Fixer - 10 minutes

3) Water - 15 minutes.

After air drying the slides they were examined by phase contrast microscopy. If cell outlines appeared difficult to determine the slides were stained in a Giemsa solution (BDH Chemicals, Toronto, Ontario).

(9) METHOD OF INFECTION FOR SEDIMENTATION ANALYSIS

Routinely 5 X 10^7 L-M cells in exponential phase of growth in suspension culture were pelleted from suspension at 1000 x g for 15 minutes at 4°C. They were resuspended in 1.0 ml of sonically treated stock vaccinia virus in medium 199P+MC. This 1.0 ml aliquot of virus had been adjusted to the appropriate titer to result in the desired multiplicity of infection (MOI). Mock-infected cells were treated similarly but re-suspended in 1.0 ml of medium 199P+MC. The mixtures were rotated at 6 rpm on a rotissary in a screw-capped 7.5 ml siliconized bijou bottle at 4°C for 1 hour for adsorption. The cells were then aseptically transferred to

a centrifuge tube. The bijou bottle was washed twice with 5 ml samples of medium, which were pooled with the original virus-cell suspension. After centrifugation to remove unadsorbed virus the cell pellet was resuspended in fresh medium to a concentration of 2-5 x 10^5 cells/ml and placed in a screw-capped Erlenmeyer shaker flask at 35°C on a gyrotory shaker incubator. This time was considered to be zero hours post-infection.

(10) INHIBITORS OF DNA, RNA, AND PROTEIN SYNTHESES

(a) INHIBITOR OF DNA SYNTHESIS

The antimetabolite chosen to inhibit DNA dependent DNA synthesis was cytosine arabinoside (cytosine-1-β-D-arabino-furanosyl, Sigma Chemical Company, St. Louis, Missouri). A 10X stock solution was made by dissolving 1 mgm in 10 ml medium 199P+MC, with stirring at 4°C, to give a concentration of 100 µgm/ml. After dissolution the material was sterilized by passing it through a 0.22 µ pore or 0.45 µ pore sterile filter.

(b) INHIBITOR OF RNA SYNTHESIS

Actinomycin D was used to inhibit DNA dependent RNA synthesis. It was obtained from the manufacturer (Calbiochem, Los Angeles, California) in 200 µgm amounts. To make a 10X stock solution 6.6 ml of medium 199P+MC was added to one of these aliquots, making a solution

with a concentration of 30 µgm/ml. Dissolution was almost instantaneous. Sterilization was achieved by filtration as above.

(c) INHIBITOR OF PROTEIN SYNTHESIS

To inhibit protein synthesis cycloheximide (actidione, K and K Laboratories, Inc., Plainview, New York) was used. A 10X stock solution was made by dissolving 1 mgm in 10 ml medium 199P+MC, with stirring at 4°C, giving a concentration of 100 µgm/ml. This material was sterilized as outlined above.

(d) METHOD OF MEASURING DEGREE OF INHIBITION

The following combinations of inhibitors and radioactive precursors were used: (i) To measure inhibition of DNA synthesis: cytosine arabinoside and ^{3}H-TdR, (ii) to measure inhibition of RNA synthesis: actinomycin D and ^{3}H-UR, and (iii) to measure inhibition of protein synthesis: cycloheximide and tritiated L-amino acid mixture. In all three cases the protocol was the same. In essence it was a repetition of that outlined in (6) above, except that now both labelled precursor and antimetabolite were present together. Control cells were treated with the radioactive material, but in place of the inhibitor was an equal volume of medium 199P+MC.

Addition of inhibitor and labelled compound took place at the beginning of the one hour pulse. If inhibitor was found to be less than 95% effective a repeat experiment was tried in which cells were pretreated with the inhibitor for an hour prior to adding the labelled compound.

(11) SIMULATED INFECTION OF L-M CELLS WITH LATEX PARTICLES

The protocol outlined in (9) above was followed exactly except for the substitution of the 1.0 ml aliquot of vaccinia virus by a 1.0 ml aliquot of a latex particle suspension. The latex particles used to simulate vaccinia virus were described as "uniform latex particles - polystyrene" (Dow Diagnostics, Indianapolis, Indiana), having a particle diameter of 0.234 μ (standard deviation 0.0026 μ). These particles were suspended in distilled water at a concentration of 1.4×10^{13} particles/ml (Dow Chemical Co., personal communication). An aliquot of the purchased material was diluted 1:300 with medium 199P+MC to give a concentration of approximately 5×10^{10} particles/ml. One ml of this material replaced the vaccinia virus aliquot in the protocol as outlined in (9) above.

(12) INACTIVATION OF VACCINIA VIRUS

(a) INACTIVATION BY ULTRAVIOLET IRRADIATION

1.5 ml of sonicated vaccinia virus, at a titer

of 3.3×10^8 pfu/ml, was placed in a sterile glass petri dish. The dish was placed, with top removed, under a 20 watt bacteriocidal ultraviolet light (General Electric, Cleveland, Ohio) at a fixed distance of 30 cm, according to the method of Faught (1974). Exposure time was 5 minutes, with periodic agitation throughout this interval.

(b) INACTIVATION BY HEAT

1.0 ml of sonicated vaccinia virus, at a titer of 6.1×10^7 pfu/ml, was placed in a bijou bottle and the bottle placed in a water bath at 57°C for 45 minutes. Periodically throughout the incubation the sample was gently agitated. A control sample of virus was kept on ice throughout the 45 minute period.

(13) NEUTRALIZATION OF VACCINIA VIRUS BY RABBIT ANTISERUM

(a) PRODUCTION OF ANTI-VACCINIA VIRUS ANTISERUM

Anti-vaccinia virus antiserum used in this study was prepared and kindly supplied by Dr. R. Mielly. It was prepared according to the following timetable of injections: On day 1, day 8, and day 15 a laboratory bred rabbit was injected subcutaneously with 0.5 ml, 0.4 ml, and 0.5 ml respectively of purified vaccinia virus in Hanks balanced salt solution. On days 71, 87, 92, and 100

injections of 1.0 ml of antigen were repeated. Finally on day 106 the animal was exsanguinated.

The blood was incubated at 37°C for 30 minutes. The serum was refrigerated overnight, and the clot which formed was rimmed and then removed. Finally the serum was decanted carefully, passed through a 0.22 μ pore sterile filter, and frozen at -90°C. The antiserum was tested by immunodiffusion against soluble antigen (sonicated infected cell extract) and was found to give 5 distinct bands.

(b) TITRATION OF ANTI-VACCINIA VIRUS ANTISERUM

Stock antiserum was titrated by the plaque reduction method. Serial ten-fold dilutions of antiserum were made in medium 199P+MC. A constant volume of a stock of vaccinia virus, of known titer, was added to each dilution of antiserum. Each mixture was incubated at 37°C for approximately 2 hours with periodic agitation. Appropriate controls were also included.

After the incubation period 1.0 ml aliquots of each sample were plated out on top of preformed confluent monolayers and incubated for plaque production, as outlined in (3) (b) above.

(c.) NEUTRALIZATION OF VIRUS PRIOR TO INFECTION OF L-M CELLS

Neutralization of 0.5 ml of vaccinia virus containing 0.7 X 10^8 pfu in medium 199P+MC was accomplished by incubating the virus with 1.0 ml of an antiserum stock (previously titrated and known to be effective in neutralizing 0.7 X 10^8 pfu) at 37°C for 1 hour in a water bath, with intermittant agitation. A control sample of virus was treated similarly, except that 1.0 ml of medium 199P+MC replaced the 1.0 ml of antiserum stock. Another control consisted of 1.0 ml of normal rabbit serum in place of antiserum.

(14) METHOD OF DETERMINING RATE OF RNA SYNTHESIS IN THE CYTOPLASM OF MOCK- or VIRUS-INFECTED L-M CELLS

The procedure used was basically that described by Metz and Esteban (1972), with some minor modifications. 0.5 ml of a stock 10 X solution of ^{3}H-UR (100 µCi/ml) was added to 4.5 ml of a suspension of mock- or virus-infected L-M cells, at various times PI, at a concentration of approximately 5 X 10^5 cells/ml. The MOI used was 10. Cells were pulsed in a bijou bottle at 37°C for 30 minutes. In later efforts the pulse was shortened to 15 minutes and the cell concentration increased to 10^6 cells/ml. The pulse was terminated by the addition of cold medium 199P+MC to

dilute out the label. Samples were centrifuged, the supernatants decanted, and the cell pellets resuspended in 5.0 ml of buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$). Samples were recentrifuged, supernatants decanted, and pellets resuspended in 2.25 ml buffer. Cells were then lysed by the addition of 0.25 ml 5% Nonidet P40. This material was centrifuged for 5-10 minutes at 800 X g. The top half of the resulting supernatant was withdrawn and considered to be the cytoplasmic material. The bottom half of the supernatant was discarded. The pellet was resuspended in 2.5 ml buffer + Nonidet P40 as above, and was considered to be nuclear material.

0.3 ml aliquots of both types of material were processed for liquid scintillation counting, as outlined in (6) (a) above, omitting the lysing step with 0.25 N NaOH. In every case a sample of the material was examined by phase contrast microscopy for cross contamination of nuclei in the cytoplasmic material. In no case were nuclei ever seen in cytoplasmic preparations.

(15) <u>DETECTION OF INTERMEDIATES IN VACCINIA VIRUS UNCOATING</u>

With significant modifications, the method

followed in detecting intermediates in vaccinia virus uncoating was that of Sarov and Joklik (1972). To 5 X 10^7 pelleted L-M suspension culture log phase cells were added 1.5 ml medium 199P+MC and 0.5 ml of ^{3}H-TdR-labelled vaccinia virus (see section 3 (c) above). The titer of the virus was 1.2 X 10^8 pfu/ml and its specific activity was 2.8 X 10^{-4} dpm/pfu. The cells and virus were mixed slowly for 45 minutes at 4°C to adsorb virus to cells. After removing unadsorbed virus the pellet of infected cells was resuspended in 75 ml fresh warmed medium 199P+MC.

At each time of interest PI a 15 ml aliquot of the suspension culture was removed for analysis. The analysis was carried out in the following manner:

The aliquot was centrifuged and the pelleted cells were washed. Then these cells were resuspended in 2.5 ml SSC (0.15 M sodium chloride, 0.015 M sodium citrate, pH 6.8) and sonicated for a total of approximately 10 seconds. Phase contrast microscopic observation assured complete cell rupture, for no field examined showed any discernible cell structures. The material could be described as "vesicle-like" only. After sonication 0.27 ml of a 10 X solution of Triton X-100 (Mann Research Laboratories, New York), 10% w/v in 10[redacted] Tris buffer (pH 8.0), 10^{-1}M KCl and 5 X 10^{-3}M 2-mercaptoethanol, was

added. The sample was then placed on ice until analyzed by ultracentrifugation.

The discontinuous gradients used for ultracentrifugation were 14 ml in volume, consisting of 2 ml each of 20%, 25%, 30%, 35%, 40%, 45%, and 50% w/w sucrose in 10^{-3}M Tris buffer (pH 8.0), 10^{-1} M KCl and 5 X 10^{-3}M 2-mercaptoethanol. The gradients were centrifuged in a SW27 rotor using SW27.1 buckets for 25 minutes at 15,000 rpm at 4°C in a model L-2 or model L2-65B ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California). 18-drop fractions were collected from the top by displacement from the bottom with 70% sucrose, using an Isco model D density-gradient fractionator (Instrumentation Specialities Co., Inc., Lincoln, Nebraska). 25% cold TCA was added to each fraction and the contents (plus 2 washings with 5% TCA) were filtered through 25 mm diameter 0.22 μ or 0.45 μ pore filters. Filters were dried and counted for radioactivity as outlined in section (6) (a) above.

RESULTS

A) PRELIMINARY EXPERIMENTS TO ESTABLISH GRADIENT CONCENTRATION RANGE

Mammalian cells of a suspension culture can be separated from each other on the basis of cell size and/or cell density differences, using the principles of sedimentation-rate separation and/or buoyant density separation. Sinclair and Bishop (1965) were the first to describe the separation of exponentially grown suspension culture strain-L mouse cells by sedimentation-rate separation. More recently Creighton (1970) described the separation of suspension culture L-M cells on a continuous Ficoll gradient. Using a 15%-22% w/v continuous Ficoll gradient she was able to separate a logarithmic phase L-M suspension culture into 3 fairly discrete subpopulations, banding at 17.2%, 18.4% and 20.4% w/v Ficoll. She reported the basis of the separation to be cell size, which had as its basis the eucaryotic cell cycle.

This observation was used as the starting point for the work described herein. L-M suspension culture cells were grown and handled as described in

Materials and Methods; in essence this was the protocol used by Creighton (1970). It was reasoned that if the L-M cells were behaving in a similar way to that found by Creighton it should be possible to separate a log phase population into 3 subclasses by subjecting the population to centrifugation on a discontinuous 17.2%: 18.4%:20.4% Ficoll gradient. Contrary to expectation, however, it was repeatedly found that centrifugation at 350 X g for 60 minutes did not result in a dispersal of cells to all 3 of the Ficoll layers; rather visual examination revealed a fairly dense population of cells uniformly distributed in the 17.2% layer, cells heavily concentrated at the 17.2%:18.4% interface, sparse numbers in the 18.4% layer, and absent from the 20.4% layer. An additional 60 minute centrifugation shifted the entire pattern down so that the 17.2% layer was now void of cells, the 18.4% layer contained uniformly distributed cells, the 18.4%:20.4% interface was heavily populated with cells, and the 20.4% layer remained void of cells. Furthermore when log phase suspension culture L-M cells were subjected to centrifugation on a 15%-22% (1.3558 - 1.3655 refractive index units) w/v continuous Ficoll gradient, only one obvious band of cells resulted from a 1 hour centrifugation following Creighton's procedure, namely at the zone having a refractive index of 1.3604 units, equivalent to 18.3% w/v Ficoll.

Two conclusions were drawn from this result: (i) the basis of the separation might not be buoyant density; (ii) the results described by Creighton (1970) could not be reproduced in this operator's hands. As a result of the latter conclusion it became apparent that Ficoll gradient concentration ranges would have to be independently established for this study.

Log phase L-M cells were concentrated by centrifuging them out of suspension and resuspending in a smaller volume of medium 199P+MC, to a final concentration of approximately 2.0×10^6 cells/ml. 1.0 ml of this cell suspension was layered onto a gradient consisting of 3.3 ml each of 15%, 19% and 22% Ficoll. The gradient was centrifuged for 55 minutes at 500 rpm in a PR2 centrifuge with a number 269 swinging bucket rotor. Results indicated that cells did not enter the 22% layer, but rather were confined to the 15% and 19% layers.

Up to this point results had not been strictly quantitated. Several changes in technique were now employed which permitted easy quantitation and minimal manipulation of cells prior to analysis. First the cell concentration step described in the paragraph above was eliminated. To obtain maximal numbers of cells for analysis without manipulation cell suspensions were grown up to concentrations of $6\text{-}7 \times 10^5$ cells/ml. Semi-log

plo[illegible] cell concentration vs. time proved that such cultures were still in the log phase of growth. The volume of a sample of cells for analysis was increased to 7.0 ml. In this way approximately 5 X 10^6 cells could be analyzed. Gradient volumes were increased to 24 ml, the centrifugal force to 1000 X g and the length of centrifugation reduced to 15 minutes. Secondly, the PR2 centrifuge and the number 269 swinging bucket rotor were replaced by the RC2B centrifuge and HB4 swinging bucket rotor. Finally fractions were collected automatically by an LKB fraction collector, by displacing the gradient upwards from the bottom with 70% sucrose, as opposed to piercing the cellulose nitrate centrifuge tube and counting drops by eyesight.

Distribution analyses using this protocol gave a pattern similar to a bell-shaped normal distribution curve. Cells were found to be located throughout at least 4/5 of the gradient, as can be seen in Figure 1. Cell recovery from gradients ranged from approximately 60% to 99%, in agreement with the results reported by Spalsbury *et al.* (1973). Any loss could be completely accounted for by cells adhering to the side walls and/or the bottom of the centrifuge tube.

It was necessary to establish the maximum concentration of Ficoll to use for optimum cell spread.

2 3

OF/DE

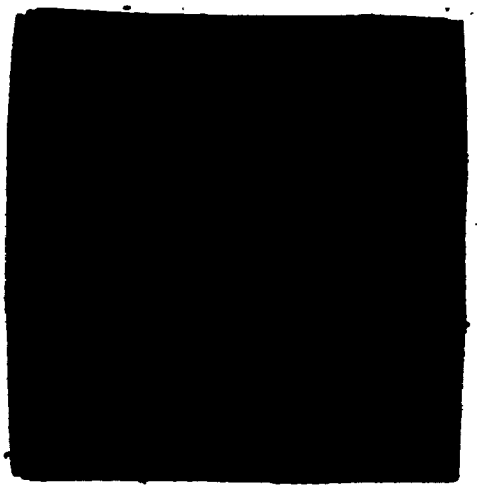


FIGURE 1

TYPICAL SEDIMENTATION DISTRIBUTION PROFILE OF LOG PHASE L-M SUSPENSION CULTURE CELLS ON A 15-19% (w/v) CONTINUOUS FICOLL GRADIENT

The procedure for loading cells on the gradient and the conditions for centrifugation were as described in Materials and Methods.

Total number of cells recovered = 33.4×10^5

Cell yield = 68%.

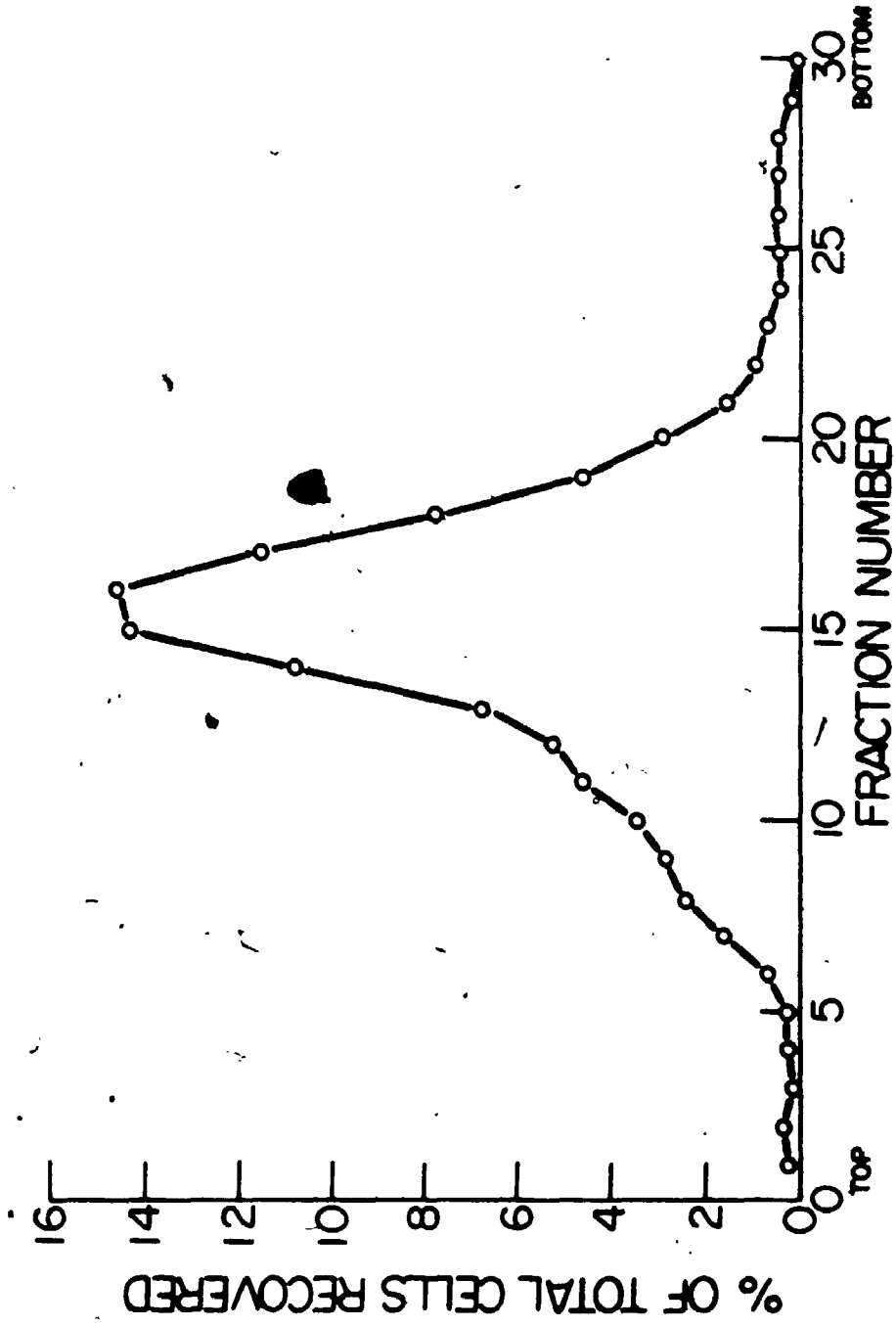

% OF TOTAL CELLS RECOVERED
0
2
4
6
8
10
12
14
16
TOP
5
10
15
20
25
30
BOTTOM
FRACTION NUMBER

FIGURE 1

It had already been established that L-M cells did not enter Ficoll concentrations greater than about 18.3%. To obtain more precise information, however, a series of continuous Ficoll gradients were constructed, with ranges of 15%-16%, 15%-17%, 15%-18%, and 15%-19%. Identical aliquots from a mother population were analyzed simultaneously. The results are plotted in Figure 2. Here it can be seen that the best overall spread of cells occurred in the gradient with a 15%-19% Ficoll range. (The low recovery of cells from the 15%-16% gradient was probably due to cells having pelleted at the bottom of the gradient tubes). Thus it was established that the best maximum concentration of Ficoll was 19% w/v.

An analogous experiment was performed to establish the best minimum concentration of Ficoll to use. Gradients of 12%-19%, 13%-19%, and 14%-19% Ficoll were employed. The results, found in Figure 3, show that the best overall spread of cells occurred in the gradient with a 14%-19% Ficoll range. Thus it was established that the best minimum concentration of Ficoll was 14% w/v. In conclusion, then, a range of 14%-19% w/v Ficoll seemed to give the best spread of L-M cells. The gradient-making device was tested for its ability to construct linear gradients by determining the refractive index values of each fraction of the gradient. Results (not

FIGURE 2

DETERMINATION OF MAXIMUM CONCENTRATION OF FICOLL FOR OPTIMUM CELL SPREAD OF LOG PHASE L-M SUSPENSION CULTURE CELLS

Loading and centrifugation conditions were as described in Materials and Methods. Cell load for each gradient = 1.8×10^6.

	Total number of cells recovered
○——○ 15-19% continuous gradient, w/v	17.5×10^5
□——□ 15-18% continuous gradient, w/v	16.0×10^5
■——■ 15-17% continuous gradient, w/v	12.5×10^5
●——● 15-16% continuous gradient, w/v	6.4×10^5

FIGURE 2

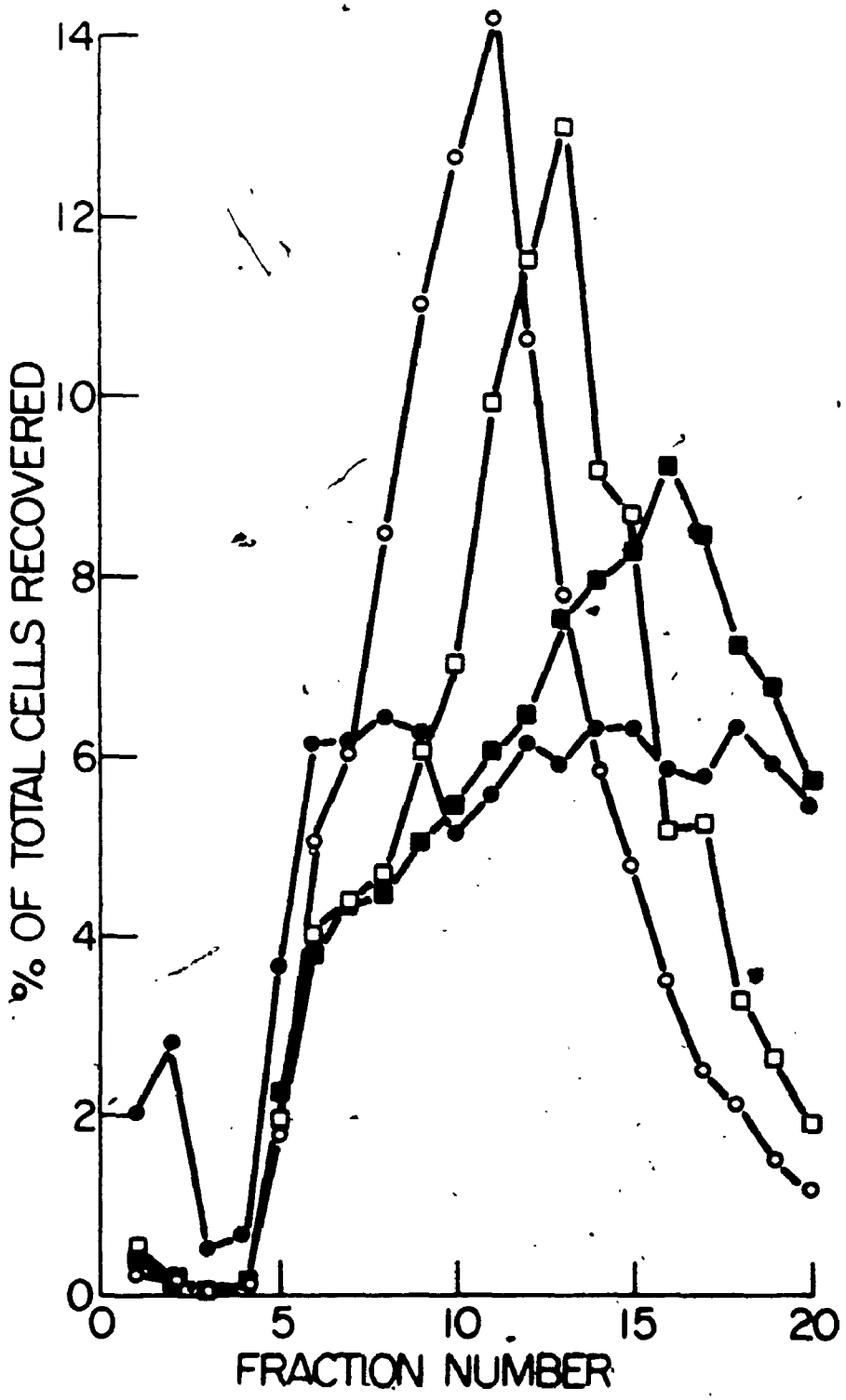

14
12
10
8
6
4
2
0
% OF TOTAL CELLS RECOVERED
0
5
10
15
20
FRACTION NUMBER

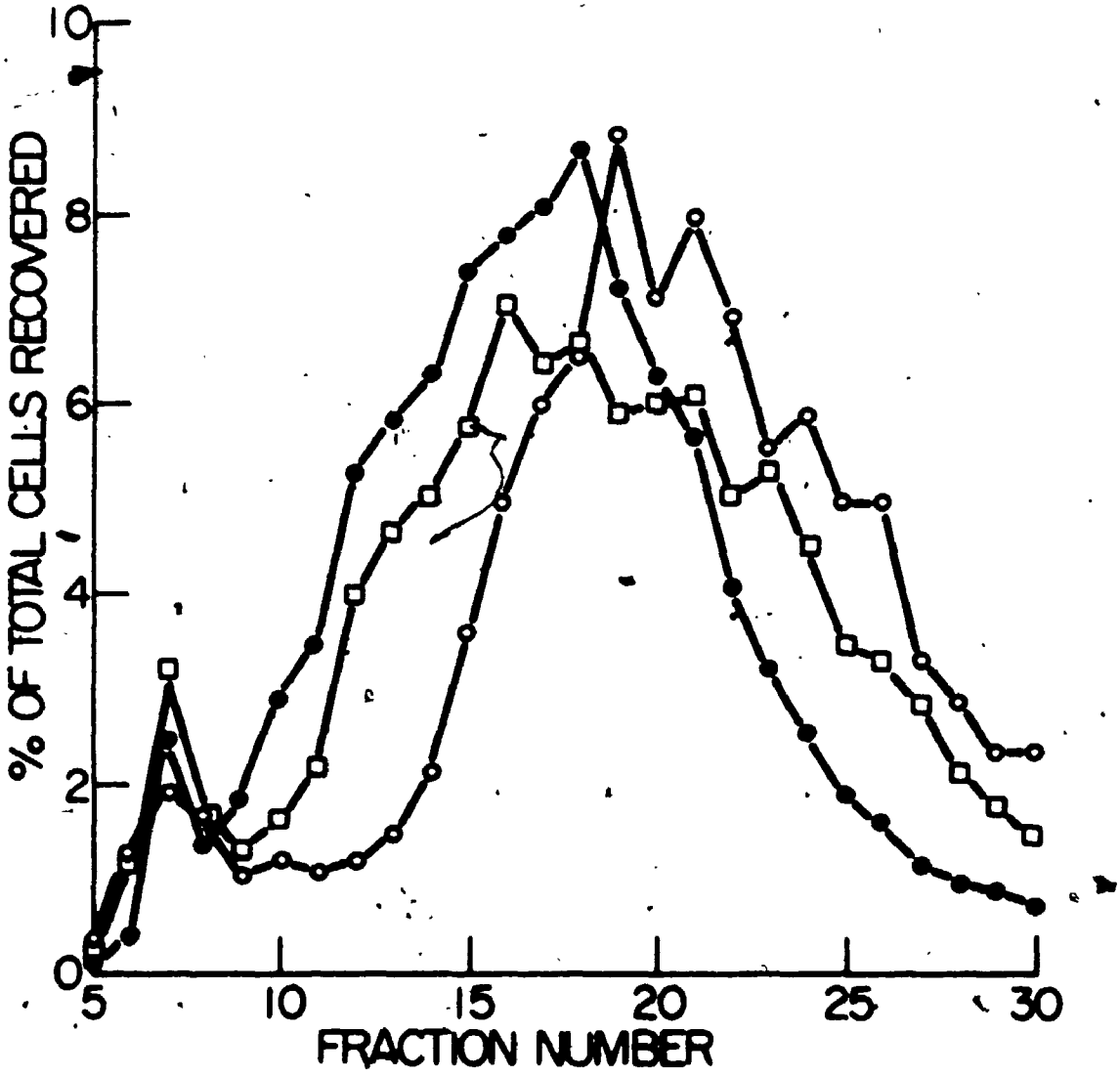
FIGURE 3

DETERMINATION OF MINIMUM CONCENTRATION OF FICOLL FOR OPTIMUM CELL SPREAD OF LOG PHASE L-M SUSPENSION CULTURE CELLS

Loading and centrifugation conditions were as described in Materials and Methods. Cell load for each gradient = 1.8×10^6.

	Total number of cells recovered
●——● 14-19% w/v continuous gradient	17.2×10^5
□——□ 13-19% w/v continuous gradient	12.3×10^5
○——○ 12-19% w/v continuous gradient	12.2×10^5

FIGURE 3


% OF TOTAL CELLS RECOVERED
0
2
4
6
8
10
5
10
15
20
25
30
FRACTION NUMBER

shown) indicated that excellent linearity existed between fraction number and refractive index value. The density of 14% Ficoll was measured to be 1.0856 gm/cc and that of 18% Ficoll 1.09020 gm/cc.

A final adjustment was made later in the routine protocol of separating log phase L-M cells on 14%-19% continuous Ficoll gradients. The adjustment was two-fold in nature. First, was the change from a continuous to a discontinuous gradient. The results of cell separations using continuous and discontinuous gradients showed no quantitative differences. The change was prompted by the greater ease involved in constructing discontinuous gradients. It was also likely that discontinuous gradients were more stable and better resisted upsets as a result of manipulation prior to, during the time of, and after centrifugation. Shortman (1972) pointed out that discontinuous gradients have limitations in their capacity to separate cells on the basis of differences in density since the only real operative part of the gradient is the zone at each interface. This criticism may be invalid for the present study since the basis of the separation is more likely cell size than cell density. Sykes *et al.* (1970) also reverted from continuous Ficoll gradients to discontinuous ones in an effort to overcome those properties of mammalian cells which these authors

felt affected satisfactory separation of cell types, namely size, density and stickiness. Therefore the concentrations used for the discontinuous gradients were 14%, 15%, 16%, 17%, 18% and 19% w/v Ficoll in medium 199P, with equal volumes of each concentration.

The second adjustment made was the elimination of the 19% Ficoll layer, based on the empirical observation that insignificant numbers of cells banded in that particular layer. This might have been due to the fact that in the case of continuous gradients (Fig. 2) any region of the gradient (other than the absolute bottom) contained Ficoll at a concentration of less than 19% w/v, whereas in the case of the discontinuous gradients there existed an entire zone of 19% Ficoll. It might have been that centrifuged cells were capable of banding in concentrations of Ficoll less than 19%, but were incapable of actually entering a zone of 19% Ficoll.

B) <u>CELL DISTRIBUTION PROFILES OF MOCK-INFECTED AND VACCINIA VIRUS-INFECTED L-M SUSPENSION CULTURE CELLS IN A FICOLL GRADIENT, AT 2 HOURS P.I. AT AN MOI = 10</u>.

The report of cellroum ding within 1-2 hours after infection of L cells by vaccinia virus (Hanafusa, 1962) prompted this investigator to examine the behaviour

of L-M suspension culture cells when infected with vaccinia virus, in terms of sedimentation properties in Ficoll gradients. Log phase suspension culture cells were infected or mock-infected at an MOI=10, as described in Materials and Methods. At 2 hours PI aliquots from each culture were analyzed for distribution profiles. Typical results can be seen in Figure 4. It was therefore concluded that by 2 hours PI vaccinia virus-infected L-M cells had demonstrated an altered sedimentation pattern. The alteration took the form of a shift of the majority of cells from a position towards the bottom of the gradient (e.g. fraction 25) to a position closer toward the top of the gradient (e.g. fraction 12). Cells in each fraction from both gradients were examined to determine if the trivial explanation of cell clumping could account for this phenomenon. In no case was cell clumping found to exist beyond background levels of 1 or 2%.

C) TEMPORAL STUDY OF THE DISTRIBUTION PROFILE SHIFT PHENOMENON

Up to this point only a static examination, at 2 hours PI, had been undertaken. To obtain information on the timing of the occurrence of this phenomenon, repetitive analyses were carried out at 0, 1, 2, and 3 hours PI, using aliquots from a single virus-infected

FIGURE 4

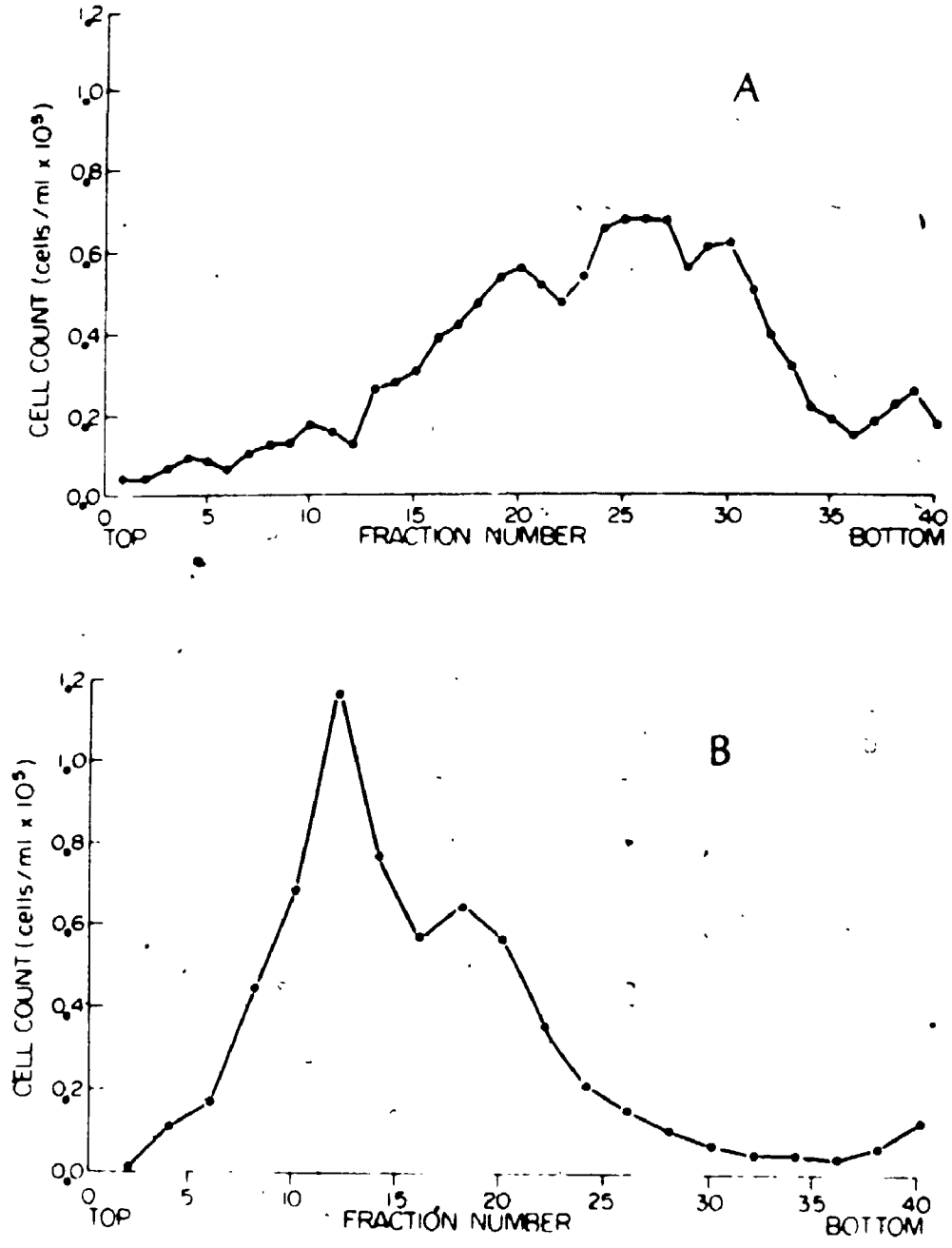
TYPICAL SEDIMENTATION DISTRIBUTION PROFILE SHIFT OF LOG PHASE L-M SUSPENSION CULTURE CELLS DUE TO INFECTION WITH VACCINIA VIRUS

Conditions for infecting, loading, and centrifuging cells were as described in Materials and Methods. The MOI in this experiment was 10. Cell load for each gradient = 12.3×10^5.

		Total number of cells recovered
A	Cell distribution at 0 hours PI	13.0×10^5
B	Cell distribution at 2 hours PI	12.9×10^5

The distribution of mock-infected cells remained as in panel A throughout the incubation period.

FIGURE 4


A
CELL COUNT (cells / ml x 10^5)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
TOP
FRACTION NUMBER
BOTTOM
B
CELL COUNT (cells / ml x 10^5)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
40
TOP
FRACTION NUMBER
BOTTOM

suspension culture. The sedimentation patterns of these cells, at an MOI = 10, can be seen in Figure 5. The mock-infected cell sedimentation pattern remained throughout as in panel A. Here it could be seen that in fact the shift in the distribution profile was time dependent. The maximum number of cells to assume the new sedimentation property did so by about 2-3 hours PI.

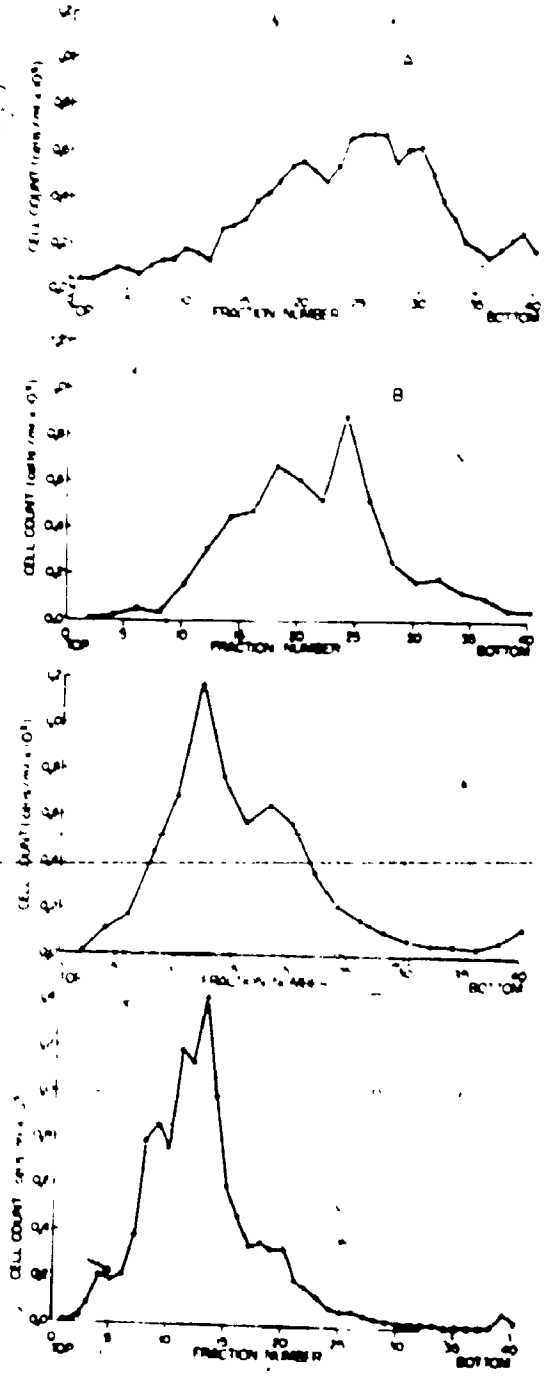
D) DEPENDENCY OF DISTRIBUTION PROFILE SHIFT ON MOI VALUE

Even at 3 hours PI it was noted that there were some small numbers of unshifted cells (Figure 5, panel D). An MOI of 10, as used in this experiment, can be shown by the Poisson distribution (Davis *et al.*, 1973) to result in 4.5 cells out of every 100,000 cells escaping infection (4.5 cells out of every 10,000 cells receive one virion, and the remainder - 99.95% of the cells - receive more than one virion). A calculation was performed on the data from Figure 5 panel D which showed that less than 1% of the unshifted cells could be explained on the basis of having escaped infection. Thus it became of some interest to learn why virtually every cell did not assume the altered sedimentation property. This point will be returned to in sections P) and Q). As a preliminary experiment, however, it was necessary to establish the effect of varying the MOI on the shift phenomenon.

of total cells recovered) of the gradient, as can be seen in Figure 11. Thus using the higher concentration of actinomycin D there appeared to be a partial response of cells to infection with vaccinia virus. For example, note in Figure 11 that near the bottom of the gradient (e.g. fractions 9-12) the drug-treated virus-infected culture assumed a value ("% of total cells recovered") intermediate between the mock-infected culture and the untreated virus-infected culture. Similarly near the top of the gradient (e.g. fractions 2-6) the drug-treated virus-infected culture assumed a value intermediate between the untreated virus-infected culture and the mock-infected culture.

Treatment of the cell culture with 10 µgm/ml actinomycin D was found to decrease incorporation of ^{3}H-UR by only about 82%, apparently therefore even less than found earlier with 3 µgm/ml of the drug. Faught (1974), using only 3 µgm/ml actinomycin D and suspension culture L-M cells, reported that incorporation of ^{3}H-UR decreased by 99%. Harry (personal communication), using 10 µgm/ml actinomycin D and confluent monolayer L-M cells, found a decrease of about 97%. The inability of this investigator to achieve these degrees of inhibition was inexplicable. However, the results found in Figure 11 allowed for the conclusion that at least some RNA synthesis was required in order to get a "typical" cell distribution shift (*viz.* almost every cell shifting in

FIGURE 5



2 hours PI was chosen as the static time for analysis since it was already known that by this time the shift phenomenon could be easily detected at MOI=10. Using 4 cultures, one mock-infected and the other 3 infected with vaccinia virus at MOI values of 0.1, 1.0 and 10.0, Ficoll gradient analyses gave the results shown in Figure 6. The gradual upward shift of cells as the MOI was raised was interpreted to mean that as more cells became infected (due to higher MOI values) more of them displayed the altered sedimentation property.

E) BIOPHYSICAL NATURE OF CELL CHANGE CAUSING ALTERED DISTRIBUTION

Most reports describing differences in the sedimentation properties of cells in density gradients have indicated that the basis of these differences was cell size and/or cell density (see Historical Review). Based on these reports it seemed likely that the basis of the altered sedimentation pattern of virus-infected cells described above was a change in cell size and/or density. Since previous results (see A above) indicated that the principle underlying the phenomenon described herein was probably not buoyant density centrifugation but rather sedimentation-rate centrifugation, it seemed more logical

FIGURE 6

EFFECT OF THE MOI ON THE CELL DISTRIBUTION PROFILE AT 2 HOURS PI

Cells were infected and analyzed on Ficoll density gradients, as described in Materials and Methods. Cell load for each gradient = 11.1×10^5.

		Total number of cells recovered
A	Distribution of mock-infected cells	8.9×10^5
B	Distribution of virus-infected cells, MOI = 0.1	7.9×10^5
C	Distribution of virus-infected cells, MOI = 1.0	8.1×10^5
D	Distribution of virus-infected cells, MOI = 10.0	8.3×10^5

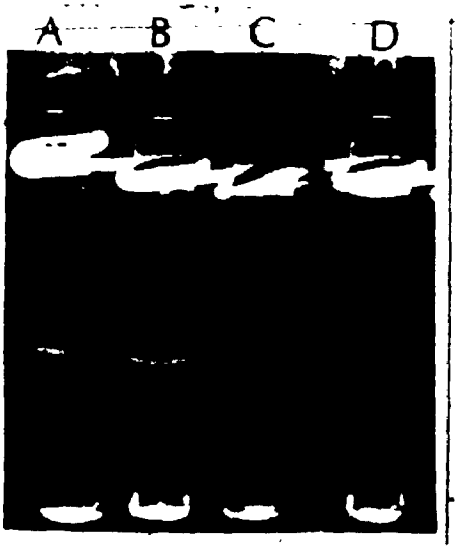
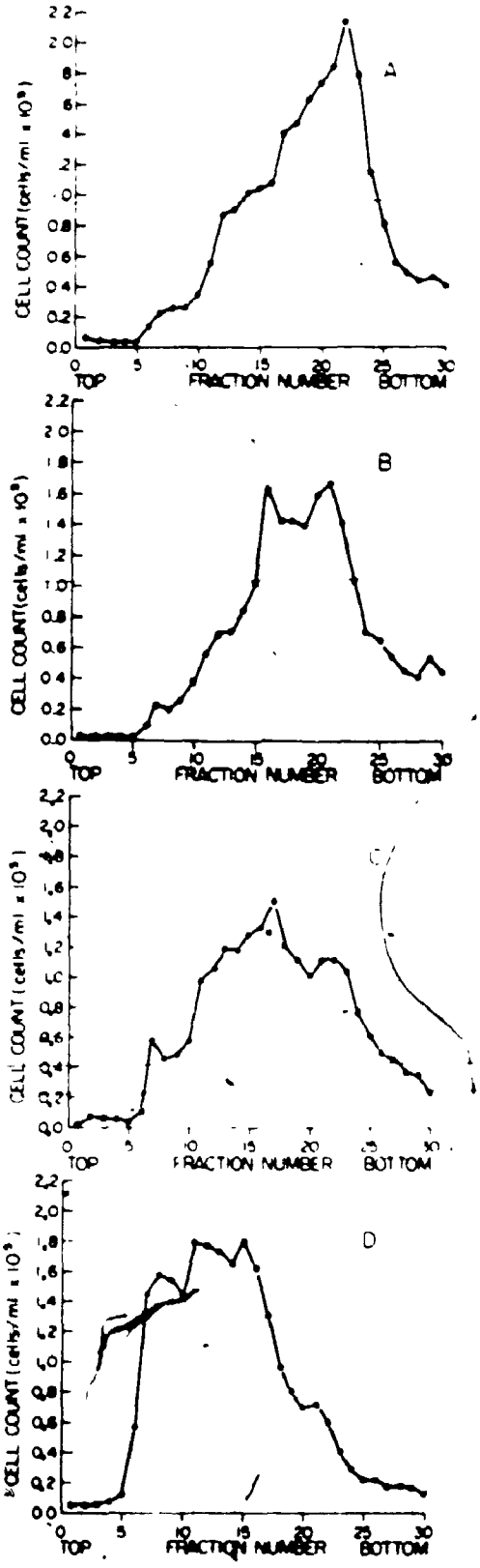


FIGURE 6



to examine cells for changes in volume ("size") rather than density, since sedimentation velocity is dependent primarily on cell size for mammalian cells (Miller and Phillips, 1969; Shortman, 1972). Ideally, infected cells should have been examined for size from every fraction of a Ficoll gradient. However, this task proved to be technically impossible to perform owing to small numbers of cells in most gradient fractions. For example, it was empirically determined that at least 10^5 cells were needed in order to generate a cell size distribution curve on a Celloscope counter linked to a pulse height channel analyzer. The Cytograf unit appeared to be about one order of magnitude more sensitive, but even with this instrument the problem of obtaining at least 10^4 cells from every gradient fraction remained relatively insoluble. A compromise was reached whereby total *unfractionated* cell populations were sized electronically. The results of such an experiment are found in Figure 7 for the Celloscope-pulse height channel analyzer system at 3 hours PI, MOI=10, and in Figure 8 for the Cytograf system at various times PI, MOI approximately 20. Relative cell size increases were only converted to absolute values in the former system. To do this 2 pollen suspensions were anlayzed on the same unit and in an identical fashion to the virus-infected and mock-infected cells. Mulberry pollen (12.5 μ mean diameter) and ragweed pollen (19.5 μ mean diameter) located as

FIGURE 7

CELLOSCOPE CELL SIZE DISTRIBUTIONS OF MOCK-INFECTED AND VIRUS-INFECTED SUSPENSION CULTURE L-M CELLS

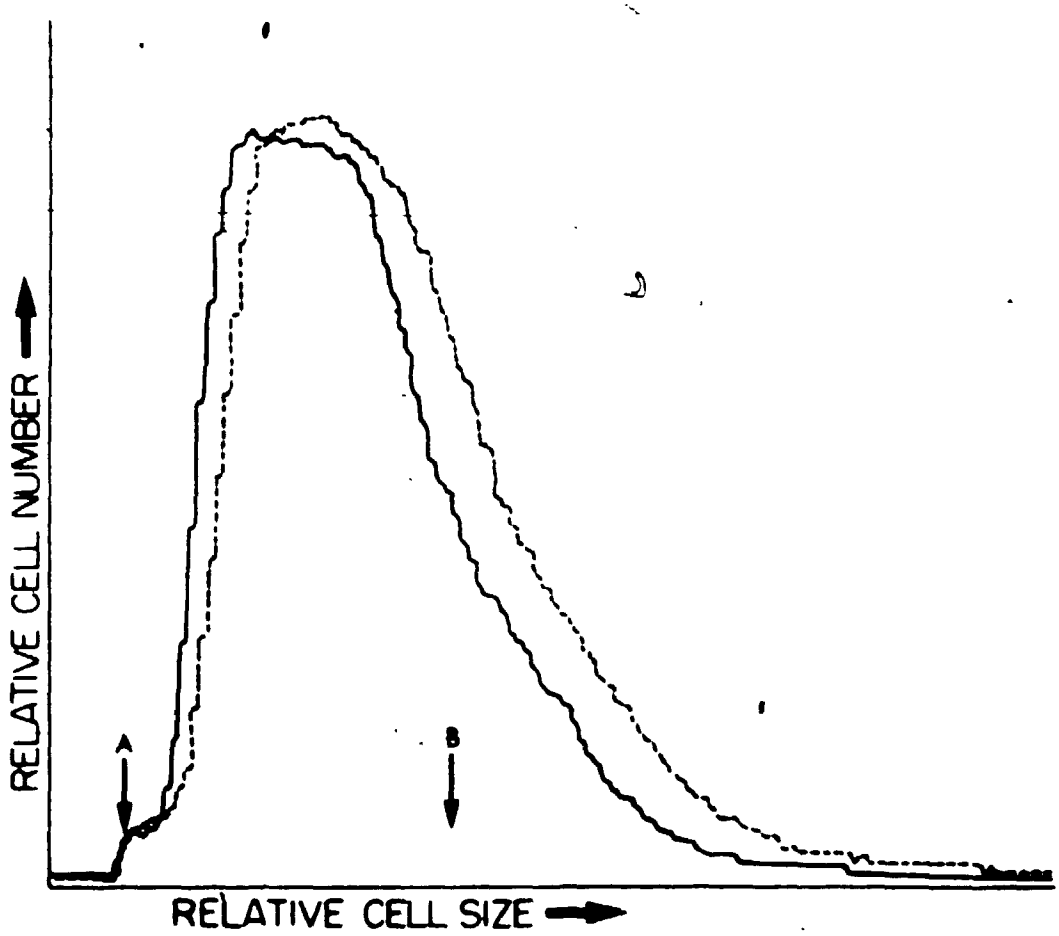
Cells were infected and electronically analyzed for size distribution as outlined in Materials and Methods.

(————————) Size distribution of mock-infected cells at 0 and 3 hours PI, and of virus-infected cells at 0 hours PI

(– – – – – – –) Size distribution of vaccinia virus-infected cells, at 3 hours PI, MOI = 10

Each curve was generated by counting at least 10^5 cells until comparable peak heights were obtained. A (arrow), mean volume ($1022\ \mu^3$) of a suspension of mulberry pollen; B (arrow), mean volume ($3880\ \mu^3$) of a suspension of ragweed pollen.

FIGURE 7



marked in Figure 7. By measuring the distance between these markers along the x axis, and knowing that this increment represented a volume change of 2858 μ^3, a mathematical relationship was established whereby a given displacement along the x axis could be converted to a given cell volume change. In this way the 3 hour mock-infected cells were measured to have a mean volume of 2745 μ^3, the corresponding diameter of which was in reasonably good agreement with the results of Creighton (1970), and the 3 hour virus-infected cells to have a mean volume of 3090 μ^3. The percent increase in the mean volume of cells as a result of infection was thus calculated to be approximately 13%, as outlined in Appendix 1. The size distributions of the mock-infected cells at 0 and 3 hr PI, and the virus-infected cells at 0 hr PI, were all identical (see legend to Figure 7).

The Cytograf data was analyzed by superimposing the histograms obtained at 1/2, 1, 1 1/2, and 2 hours PI, MOI approximately 20 (Figure 8). In this way it could be seen that cell size increased after virus infection, and further that this was a time dependent phenomenon, steadily increasing from 1/2 to 2 hours PI. (The mock-infected cells appeared to increase in size only slightly, if at all, between 0 & 2 hr PI). These results were therefore in excellent agreement with those reported in section C)

FIGURE 8

CYTOGRAF CELL SIZE DISTRIBUTIONS OF MOCK-INFECTED AND VIRUS-INFECTED SUSPENSION CULTURE L-M CELLS

Cells were infected and electronically analyzed for size distribution as outlined in Materials and Methods.

A Size distribution of virus-infected cells, 1/2 hour PI, MOI = 20

B Size distribution of virus-infected cells, 1 hour PI, MOI = 20

C Size distribution of virus-infected cells, 1 1/2 hours PI, MOI = 20

D Size distribution of virus-infected cells, 2 hours PI, MOI = 20

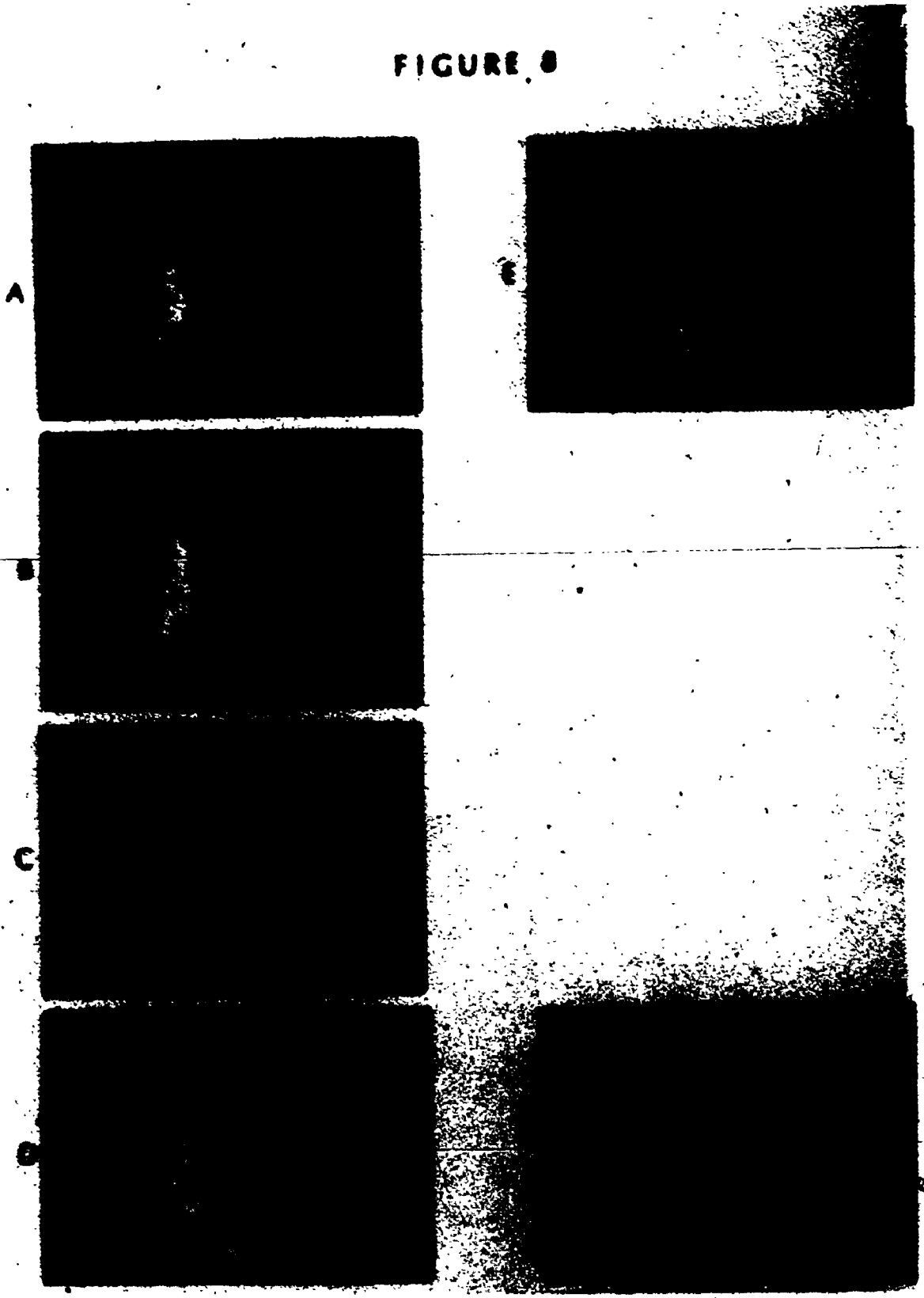
E Size distribution of mock-infected cells, 1/2 hour PI

F Size distribution of mock-infected cells, 2 hours PI

Conventional x axis - amplitude of A sensor pulse (see Appendix 6)

Conventional y axis - number of pulses per channel

FIGURE 8



above.

These data indicated then that cell size did change after infection, the change being a volume increase up to 20%. It could only be assumed, however, that the cell volume increase corresponded to the sedimentation profile shift in the Ficoll gradient.

F) LOCATION IN A FICOLL GRADIENT OF INFECTED MEMBERS OF THE CELL POPULATION

From the results of section D) it was theorized that cells which assumed the altered sedimentation property did so because they had become infected with vaccinia virus. To see if this was in fact that correct explanation an infected cell population was fractionated on a Ficoll gradient, and the cells in each fraction were examined for their infectivity. To do this an MOI less than 1 was required, for only under conditions in which statistically not every cell became infected with a virion would a chance exist to separate infected from uninfected cells. An MOI= 0.1 was chosen, which at 2 hours PI was known to result in a negligible shift in the gradient profile (see Figure 6). Aliquots from mock-infected and vaccinia virus-infected cells were analyzed on sterile gradients. Cell counts and

infectious center assays were performed on samples from each fraction. The results are plotted in Figure 9. The data was treated to show that, although all cells infected with virus did not locate in the upper fractions (fractions 2-4), nor conversely did all cells which escaped infection locate in the lower fractions (fractions 5-8), nevertheless a trend did exist whereby a four-fold greater number (44% vs. 11%) of virus producing cells (the infected sub-population) were found in the upper region of the gradient (fraction 3) than in the lower region of the gradient (fraction 5). This was found even though the vast majority of cells located in the lower region. As a check on the MOI effectively achieved (from the Poisson distribution an MOI of 0.1 should lead to just under 10% of all cells becoming infected), a calculation was performed on the above data to determine the percent of those cells recovered from the gradient which were virus producers (i.e. were themselves infected). Such a calculation showed that in one case 2.4% and in another case 12.6% of all cells recovered were actually infected. Hence the MOI effectively achieved was very close to that calculated from theoretical considerations.

Thus the data indicated an enrichment of infected members of the population in a region of the gradient nearer the top, rather than where the majority of the cell population banded. In a parallel experiment

FIGURE 9

LOCATION OF INFECTED CELLS IN A FICOLL GRADIENT AT 2 HOURS PI, AT AN MOI OF 0.1

Cells were infected and analyzed aseptically on sterile Ficoll gradients, as described in Materials and Methods. Each 1-ml fraction was assayed for cell count (O- - - - -O) and for plaque-forming cells (x- - - - -x). From these data, the percent of plaque-forming cells in each fraction (●——●) was calculated.

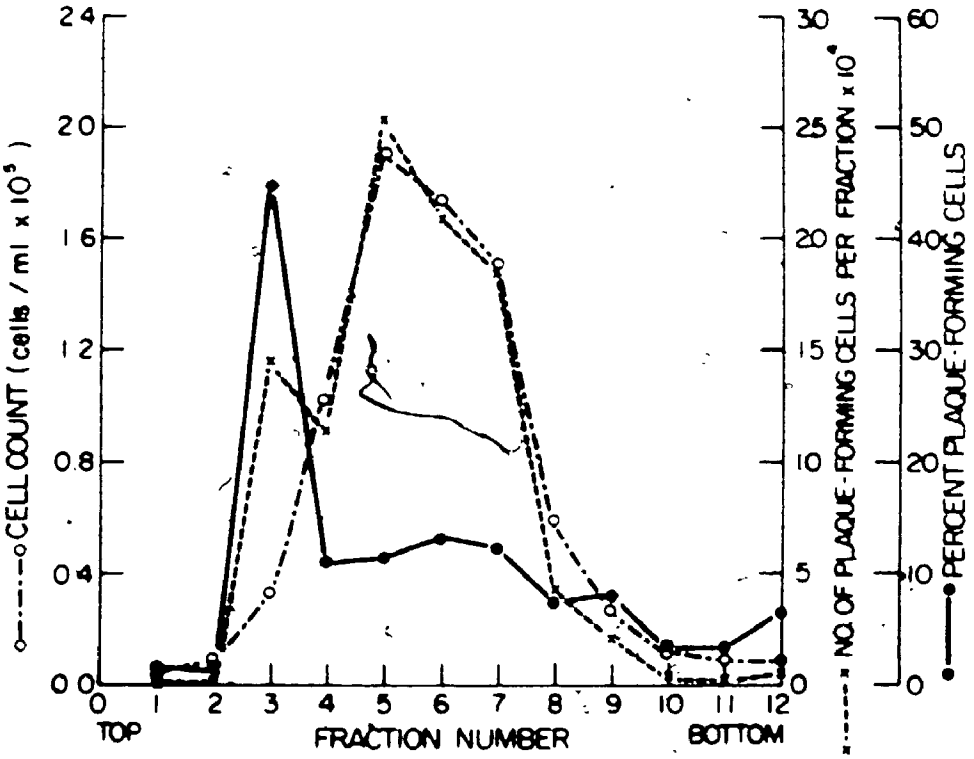
The cell count curve of the mock-infected population was omitted from this figure in order to avoid cluttering the graph. It was virtually identical in shape and position to the cell count curve of the virus-infected population (O- - - - -O), except for a possible displacement in the peak fraction, which was found in fraction 6 in the mock-infected culture vs. fraction 5 in the virus-infected culture.

Load of cells on gradient = 8.7×10^5

Total number of cells recovered from gradient = 7.8×10^5

Therefore total recovery = 89%

FIGURE 9


CELL COUNT (cells / ml x 10⁵)
NO. OF PLAQUE-FORMING CELLS PER FRACTION x 10⁴
PERCENT PLAQUE-FORMING CELLS
FRACTION NUMBER
TOP
BOTTOM
0 0
0 4
0 8
1 2
1 6
2 0
2 4
0
5
10
15
20
25
30
0
10
20
30
40
50
60
0 1 2 3 4 5 6 7 8 9 10 11 12

the virus-infected cell culture was analyzed at both 2 and 8 hours PI. It was found (results not shown) that the percentage of virus-producing cells in a *pool* of fractions 1-4 increased from 2 to 8 hours PI, (from 13% to 25%, resp.) with a concomitant decrease in the percentage of virus-producing cells in fraction 5 (from 35% to 14%, resp.).

G) EFFECT OF CYTOSINE ARABINOSIDE, ACTINOMYCIN D AND CYCLOHEXIMIDE ON DNA, RNA AND PROTEIN SYNTHESES, RESPECTIVELY

As a preliminary to determining the effect of the above-mentioned antimetabolites on the occurrence of the distribution profile shift phenomenon it was necessary to determine the effectiveness of these compounds in inhibiting certain macromolecular syntheses.

To test for inhibition of DNA synthesis an aliquot of L-M cells was pulsed for one hour with 1uCi/ml ^{3}H-TdR in the presence of 10 µgm/ml ara C, as outlined in Materials and Methods. This concentration of inhibitor was reported to have inhibited DNA synthesis in L cells by at least 99% (Oda and Joklik, 1967). At the end of the hour of incubation it was apparent from the results that the inhibited culture had incorporated approximately 1-5% of the ^{3}H-TdR incorporated in the uninhibited culture.

Inhibition of RNA synthesis was tested in an analogous fashion, using 5 µCi/ml or 10 µCi/ml of ^{3}H-UR together with actinomycin D at a final concentration of 3 µgm/ml. McAuslan (1963), using a conc. of this inhibitor only slightly greater (5 µgm/ml), reported the complete inhibition of RNA synthesis in HeLa cells within 30 minutes. At the end of an hour's incubation results indicated that the inhibited culture had still incorporated up to 10% of the ^{3}H-UR incorporated in the uninhibited culture. To reduce this value to approximately 1-5% it was found that the cells had to be incubated with actinomycin D for one hour prior to the hour of pulsing.

Inhibition of protein synthesis was tested in an analogous fashion, using 1 µCi/ml of a mixture of tritiated L-amino acids, and cycloheximide at a final concentration of 10 µgm/ml. Results indicated that at the end of a one hour pulse the inhibited culture had incorporated only 1-5% of the amount of radioactivity incorporated in the uninhibited culture.

Prior to determining the effect of these antimetabolites on the occurrence of the distribution shift phenomenon it was necessary to know the secondary effects of these inhibitors. T. Harry (personal communication), using identical techniques, found that

ara C inhibited protein synthesis by 40% when inhibitor and tritiated amino acid mixture were present together for either 1 or 4 hours. Actinomycin D was found to inhibit protein synthesis by 15% at 2 hours.

The final preliminary experiment was designed to determine whether the 3 inhibitors being used were as effective in inhibiting macromolecular syntheses in vaccinia virus-infected cells as they were in causing inhibition in uninfected cells. Results (not shown) indicated that in all 3 cases incorporation of the respective radioactive precursor was reduced by the drug as much in infected cells as in uninfected cells.

H) (i) EFFECT OF INHIBITING DNA SYNTHESIS ON OCCURRENCE OF DISTRIBUTION PROFILE SHIFT PHENOMENON

In an effort to understand the events at the molecular level taking place within the infected cell which led to the sedimentation profile shift, a series of experiments were performed in which infected cells were treated with different antimetabolites. The mode of action of each of these drugs was known, so that results could be meaningfully interpreted. In separate experiments antimetabolites were used to inhibit DNA synthesis, RNA synthesis, and protein synthesis.

To investigate the requirements for DNA synthesis for the sedimentation profile shift to occur, an experiment was performed in which cultures of mock- and virus-infected L-M cells with and without ara C were analyzed at 2 hours PI on standard Ficoll gradients. Ara C is known to inhibit the multiplication of DNA viruses by interfering with DNA synthesis. Apparently this analog of cytosine inhibits the reduction of cytidylic to deoxycytidylic acid (Davis *et al.*, 1973).

Typical results are found in Figure 10. Parallel cultures indicated that DNA synthesis had been inhibited by over 95%. These results were taken to mean that DNA dependent DNA synthesis was not required for vaccinia virus to induce the change in the distribution profile of L-M cells in Ficoll gradients.

(ii) EFFECT OF INHIBITING RNA SYNTHESIS ON OCCURRENCE OF DISTRIBUTION PROFILE SHIFT PHENOMENON

To investigate the requirement for RNA synthesis for the sedimentation profile shift to occur, an experiment was performed in which cultures of mock- and virus-infected L-M cells with and without 3 µgm/ml actinomycin D were analyzed at 2 1/2 hours PI, MOI = 10, on standard Ficoll gradients. Actinomycin D is known to bind to helical DNA at GC pairs and in this way inhibit

FIGURE 10

EFFECT OF INHIBITING DNA SYNTHESIS ON CELL DISTRIBUTION PROFILE SHIFT AT 2.5 HOURS PI, AT AN MOI OF 10

Cells were treated with cytosine arabinoside, infected, and analyzed on Ficoll gradients as outlined in Materials and Methods.

		Estimated cell yield
A	Distribution of mock-infected cells in presence of ara-C	90%
B	Distribution of mock-infected cells in absence of ara-C	90%
C	Distribution of virus-infected cells in presence of ara-C	90%
D	Distribution of virus-infected cells in absence of ara-C	90%

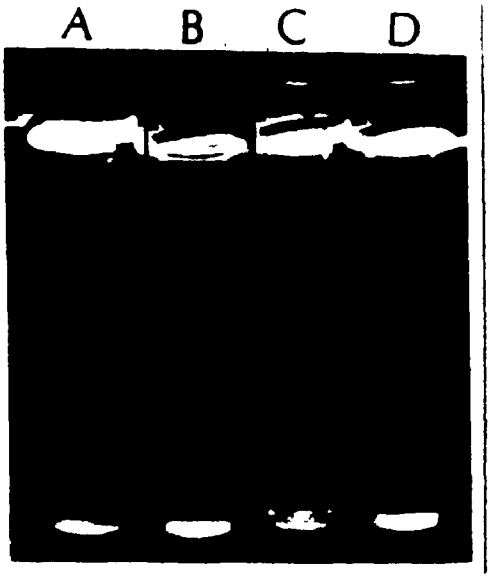
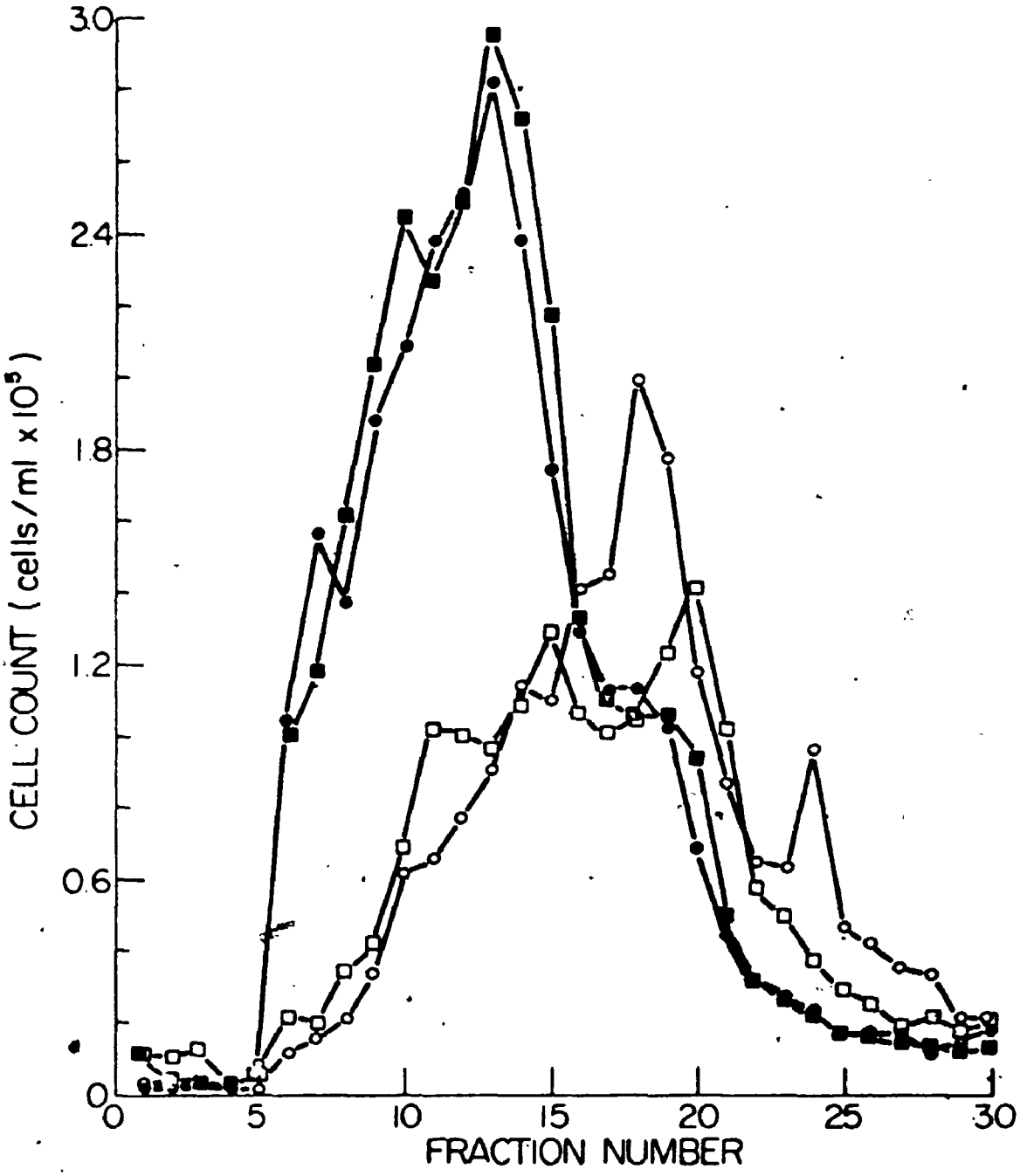


FIGURE 10


3.0
2.4
1.8
1.2
0.6
0
CELL COUNT (cells/ml x10⁵)
0
5
10
15
20
25
30
FRACTION NUMBER

extension of the RNA chain being transcribed from the DNA by the DNA dependent RNA polymerase enzyme. Part of the molecule of the antibiotic intercalates into the helix of the DNA, and the other part appears to bind to the external surface of the DNA. (Davis *et al.*, 1973).

Typical results (not shown) were rather ambiguous in that it appeared that virus-infected cells in the presence of the inhibitor assumed an intermediate position in the gradient between the shifted cells (virus-infected in absence of inhibitor) and unshifted cells (mock-infected in presence or absence of inhibitor). Since the degree of inhibition of incorporation of ^{3}H-UR into TCA precipitable material in parallel cultures was only approximately 90%, the experiment was repeated using 10 µgm/ml actinomycin D. Concomitantly an experiment was designed to determine the degree of inhibition of viral specific mRNA synthesis in the cytoplasm of vaccinia virus-infected cells in the presence of 3 µgm/ml and 10 µgm/ml actinomycin D (see I below).

Using 10 µgm/ml actinomycin D, at an MOI = 10, it was found at 2 1/2 hours PI that cells distributed themselves in a standard Ficoll gradient throughout the entire gradient, with some small peaks near the top (9% of total cells recovered) and near the bottom (12%

of total cells recovered) of the gradient, as can be seen in Figure 11. Thus using the higher concentration of actinomycin D there appeared to be a partial response of cells to infection with vaccinia virus. For example, note in Figure 11 that near the bottom of the gradient (e.g. fractions 9-12) the drug-treated virus-infected culture assumed a value ("% of total cells recovered") intermediate between the mock-infected culture and the untreated virus-infected culture. Similarly near the top of the gradient (e.g. fractions 2-6) the drug-treated virus-infected culture assumed a value intermediate between the untreated virus-infected culture and the mock-infected culture.

Treatment of the cell culture with 10 µgm/ml actinomycin D was found to decrease incorporation of ^{3}H-UR by only about 82%, apparently therefore even less than found earlier with 3 µgm/ml of the drug. Faught (1974), using only 3 µgm/ml actinomycin D and suspension culture L-M cells, reported that incorporation of ^{3}H-UR decreased by 99%. Harry (personal communication), using 10 µgm/ml actinomycin D and confluent monolayer L-M cells, found a decrease of about 97%. The inability of this investigator to achieve these degrees of inhibition was inexplicable. However, the results found in Figure 11 allowed for the conclusion that at least some RNA synthesis was required in order to get a "typical" cell distribution shift (*vis.* almost every cell shifting in

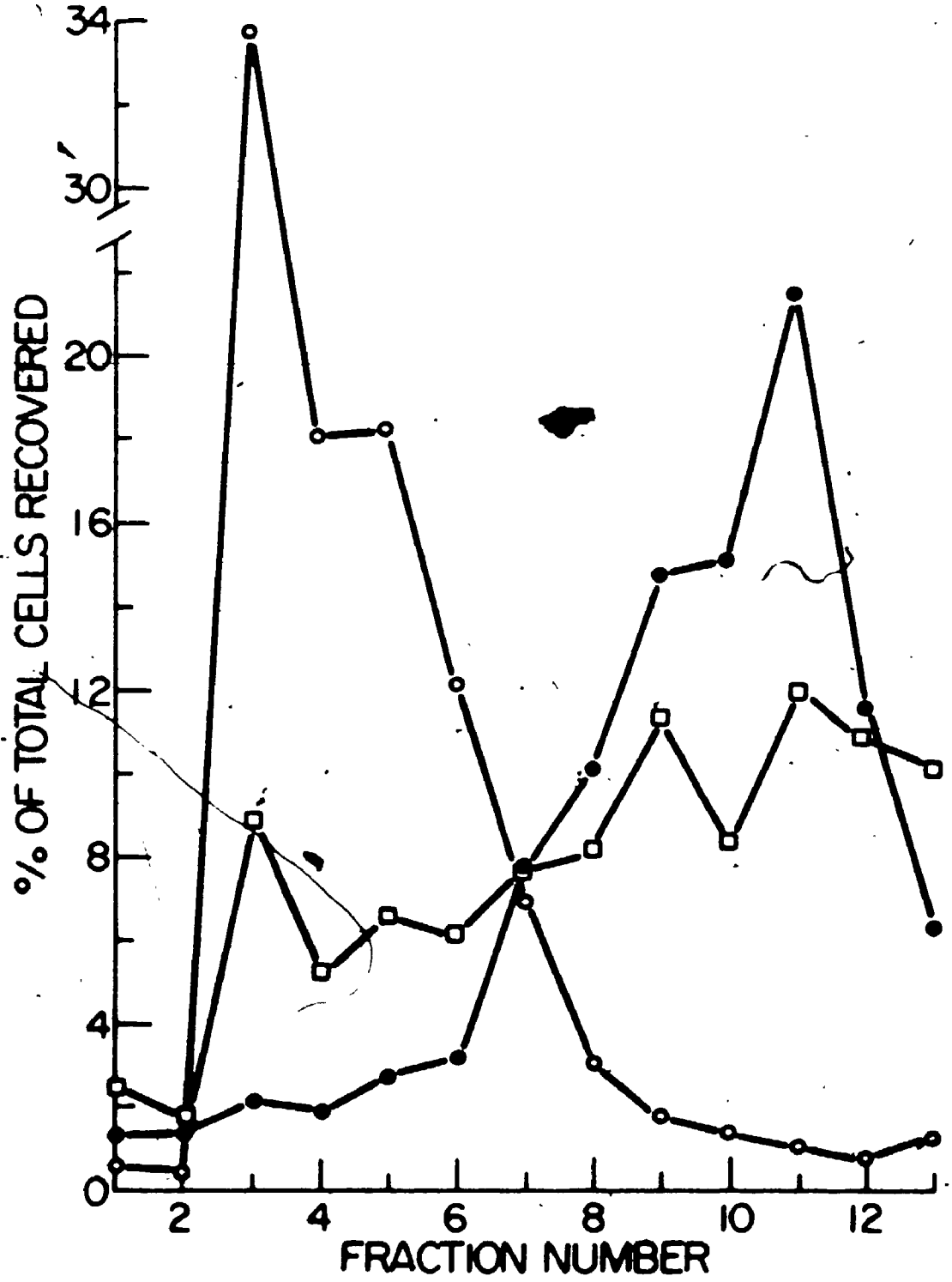
FIGURE 11

EFFECT OF INHIBITING RNA SYNTHESIS ON CELL DISTRIBUTION PROFILE SHIFT AT 2.5 HOURS PI, AT AN MOI OF 10

Cells were treated with actinomycin D, infected, and analyzed on Ficoll gradients, as outlined in Materials and Methods. Cell load for each gradient = 8×10^5 (approx.)

		Total number of cells recovered
○——○	Distribution of virus-infected cells in absence of actinomycin D	7.2×10^5
□——□	Distribution of virus-infected cells in presence of actinomycin D	4.0×10^5
●——●	Distribution of mock-infected cells in absence (or presence) of actinomycin D	3.4×10^5

FIGURE 11


34
30
20
16
12
8
4
0
% OF TOTAL CELLS RECOVERED
2
4
6
8
10
12
FRACTION NUMBER

position). Furthermore, if the poor cell recoveries of the virus-infected drug-treated culture & the mock-infected cultures (see legend to Fig. 11) were due to cells pelleting onto the bottom of the gradient tube, then in fact these 2 curves should be skewed even more than they are towards the bottom. If this were the case then the conclusion drawn above would have an even stronger basis.

(iii) EFFECT OF INHIBITING PROTEIN SYNTHESIS ON OCCURRENCE OF DISTRIBUTION PROFILE SHIFT PHENOMENON

To investigate the requirement for protein synthesis for the sedimentation profile shift to occur, an experiment was performed in which cultures of mock- and virus-infected L-M cells with and without cycloheximide were analyzed at 2 1/4 hours PI, MOI = 10, on standard Ficoll gradients. Cycloheximide inhibits protein synthesis by blocking the transfer of activated amino acids, on the ribosome, from transfer RNA to growing polypeptide chains (Davis *et al.*, 1973).

Typical results are found in Figure 12. Parallel cultures indicated that protein synthesis had been inhibited by approximately 95%. These results were taken

FIGURE 12

EFFECT OF INHIBITING PROTEIN SYNTHESIS ON CELL DISTRIBUTION PROFILE SHIFT AT 2.5 HOURS PI, AT AN MOI OF 10

Cells were treated with cycloheximide, infected, and analyzed on Ficoll gradients, as outlined in Materials and Methods. Cell yields ranged from 77% (C) to 100% (A and D).

		Total number of cells recovered
A	Distribution of mock-infected cells in presence of cycloheximide —■—	30.1×10^5
B	Distribution of mock-infected cells in absence of cycloheximide —□—	28.5×10^5
C	Distribution of virus-infected cells in presence of cycloheximide —○—	23.4×10^5
D	Distribution of virus-infected cells in absence of cycloheximide —●—	30.3×10^5

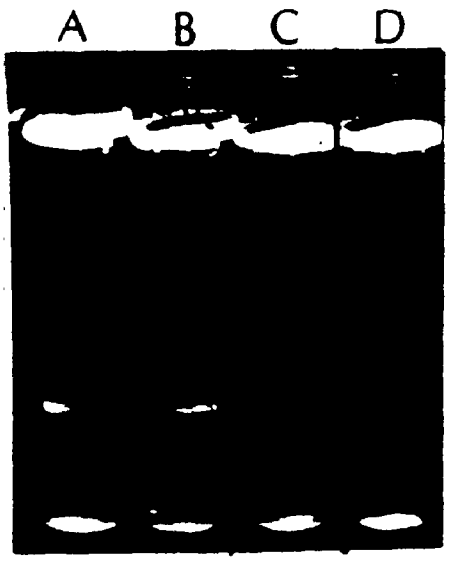
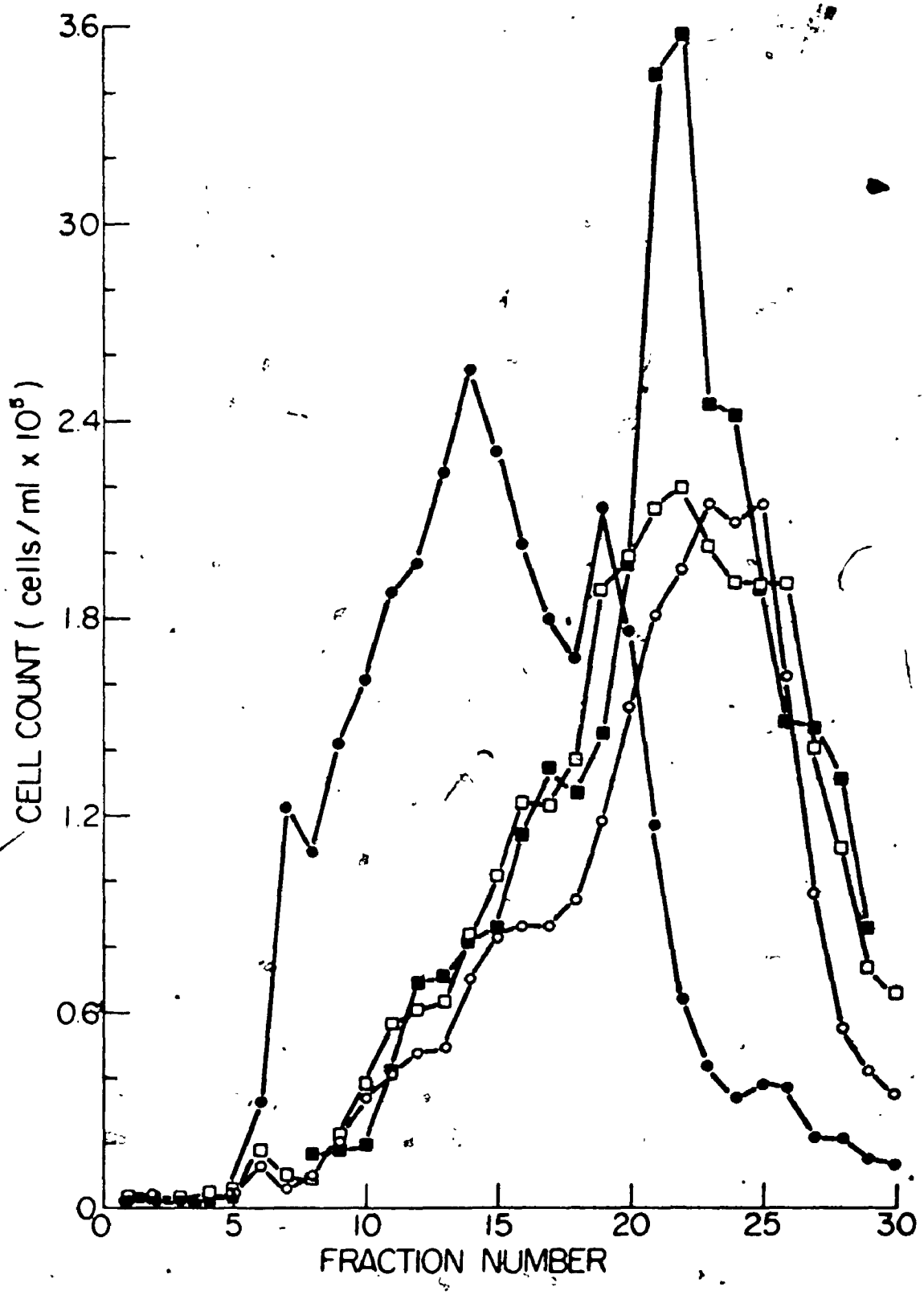


FIGURE 12


3.6
3.0
2.4
1.8
1.2
0.6
0
CELL COUNT (cells / ml x 10^5)
0
5
10
15
20
25
30
FRACTION NUMBER

to mean that protein synthesis was required for vaccinia virus to induce the change in the distribution profile.

Taken collectively, the data obtained from the experiments described in this section indicated that RNA and protein syntheses were required in vaccinia virus-infected L-M cells in order that the Ficoll gradient sedimentation shift occur, and that DNA synthesis was not required.

I) DETECTION OF SYNTHESIS OF VIRAL SPECIFIC MESSENGER RNA (mRNA) IN THE CYTOPLASM OF INFECTED CELLS

The results found in H) (ii) above, namely that 3 µgm/ml & 10 µgm/ml actinomycin D might have been allowing for the synthesis of some vaccinia virus specific mRNA, prompted an investigation into the effectiveness of 3 µgm/ml and 10 µgm/ml of this inhibitor in preventing the cytoplasmic transcription of viral specific mRNA from parental virus DNA. Because vaccinia virus replicates in the cell cytoplasm (see Historical Review) it can be assumed that any increase in RNA synthesis in the cytoplasm of cells after virus infection represents virus specific RNA synthesis.

Following the procedure of Metz and Esteban (1972) with minor modifications as outlined in Materials and Methods, control cultures were first examined i.e. mock- and virus-infected cultures in the *absence* of

actinomycin D. On only the first attempt (see Figure 13), out of a total of five, did the results agree with those reported by Metz and Esteban (1972). The results presented in figure 13 indicated that a spike of cytoplasmic RNA synthesis occurred at approximately 60 minutes PI in the cytoplasm of the vaccinia virus-infected cells. The cytoplasm of the mock-infected cells, as well as the nuclei of both mock- and virus-infected cells, exhibited no such activity. Note that by 2 hours PI significant TCA insoluble material was present in the cytoplasm of mock-infected cells. This was believed to possibly represent the appearance of cell specific mRNA which had been synthesized in the nucleus and then migrated out into the cytoplasm for translation. However since no appreciable increase in RNA synthetic activity was detected in the nuclei of these mock-infected cells *prior* to 2 hours PI, this explanation may not be correct. Furthermore, according to Becker and Joklik (1964), appreciable amounts of newly synthesized cellular RNA larger than 4S are not transported from the nucleus to the cytoplasm during a pulse of 20 minutes or less with radioactive uridine.

In any case, on four other occasions when attempts were made to repeat this finding, the results of the virus-infected cell cytoplasms were paralleled

FIGURE 13

KINETICS OF RNA SYNTHESIS IN L-M CELLS

Cells were infected with vaccinia virus, pulsed with ^{3}H-UR, and separated into cytoplasmic and nuclear material, as described in Materials and Methods.

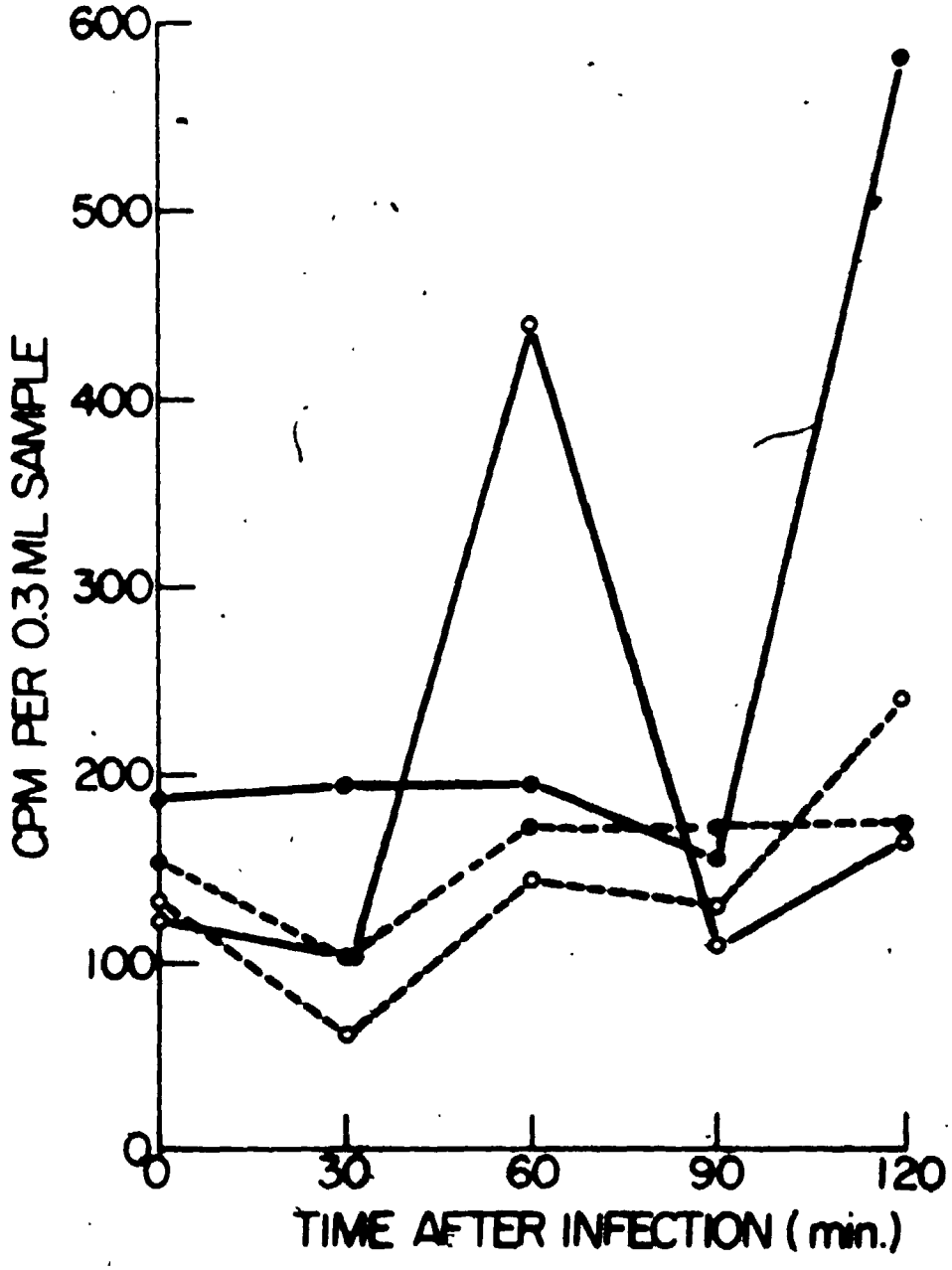

RNA synthesis in mock-infected cell cytoplasm


RNA synthesis in mock-infected cell nucleus


RNA synthesis in virus-infected cell cytoplasm


RNA synthesis in virus-infected cell nucleus

FIGURE 13


600
500
400
300
200
100
0
CPM PER 0.3 ML SAMPLE
0
30
60
90
120
TIME AFTER INFECTION (min.)

throughout by the mock-infected cell cytoplasms. This prompted a check on the efficacy of Metz and Esteban's technique in this investigator's hands. In this check cells were treated as outlined in Materials and Methods except that ^{3}H-UR was replaced by ^{3}H-TdR, the rationale for the replacement being that newly synthesized DNA should remain completely in the cell nucleus. Results (not shown) clearly indicated that such was the case, for the cell cytoplasm was found to contain only 1% of the radioactivity incorporated in the nucleus.

The conclusion drawn was that Metz and Esteban's technique was successful in this study in clearly separating nucleus from cytoplasm, but the reason for the inability of this operator to routinely detect viral specific mRNA synthesis in the cytoplasm of virus-infected cells was unknown. Consequently the effectiveness of 3 µgm/ml and 10 µgm/ml actinomycin D in preventing the cytoplasmic transcription of viral specific mRNA from parental virus DNA could not be determined.

J) EFFECT OF UV-INACTIVATED VACCINIA VIRUS ON OCCURRENCE OF DISTRIBUTION PROFILE SHIFT PHENOMENON

Joklik (1964) found that exposure of rabbitpox virus to 160 seconds of U-V irradiation, which

reduced infectivity by more than 3 logs in value, resulted in only a small decrease (9%) in the percent of virus particles converted to cores (primary uncoating), but a drastic decrease (97%) in the conversion of viral DNA from a DNAase insensitive state to a DNAase sensitive state (secondary uncoating). Joklik explained these findings on the basis that U-V irradiation inactivated the "viral inducer protein" thereby inhibiting secondary uncoating.

A more up-to-date interpretation of these findings might be that U-V irradiation inactivated the virion-bound DNA dependent RNA polymerase, which presumably codes for an uncoating protein (Joklik, 1968). For example, Bakay and Burke (1972) found that a 5 minute U-V irradiation exposure of vaccinia virus resulted in a decrease in activity of this enzyme by approximately 90%. This same exposure time resulted in complete loss in infectivity. However it should also be borne in mind that the prime target for U-V irradiation of vaccinia virions is considered to be the nucleic acid (Sime and Bedson, 1973).

To see if the inhibition of secondary uncoating prevented the occurrence of the typical cell sedimentation distribution profile shift, L-M suspension culture cells

were infected with U-V-inactivated vaccinia virus. In more detail, three cultures of L-M cells were used in this experiment. One culture was mock-infected, one was infected with U-V-irradiated vaccinia virus, and one was infected with unirradiated vaccinia virus, MOI = 10. The volume of irradiated vaccinia virus used (see Materials and Methods) was equal to that used in the unirradiated control. Irradiation was found to decrease the titer by more than 99%; thus the effective MOI of the irradiated virus-LM cell system was less than 0.1.

Cultures were analyzed at 2 hours PI on standard Ficoll gradients. Results are found in Figure 14. Here it can be seen that U-V-inactivated vaccinia virus did not induce the characteristic change in the distribution profile of L-M cells which the unirradiated virus did.

The tentative conclusion drawn from these results was that inhibition of secondary uncoating prevented vaccinia virus from inducing L-M cells to redistribute themselves in Ficoll density gradients. This was not to say that the act of secondary uncoating itself was necessary for the occurrence of the shift, however, for U-V irradiation may only prevent secondary uncoating by interfering with certain steps in the virus replication cycle, one of which leads up to the event of secondary uncoating itself. Therefore it is equally possible that

FIGURE 14

EFFECT OF ULTRA-VIOLET-INACTIVATED VACCINIA VIRUS ON CELL DISTRIBUTION PROFILE SHIFT AT 2 HOURS PI, AT AN MOI OF 10

Virus was U-V-inactivated, cells were infected, and cells were analyzed on Ficoll gradients, as outlined in Materials and Methods. Cell yields were: (A) 64%; (B) 97%; (C) 72%.

		Total number of cells recovered
A	Distribution of cells infected with U-V-inactivated vaccinia virus —○—	5.9×10^5
B	Distribution of cells infected with unirradiated vaccinia virus —□—	9.6×10^5
C	Distribution of mock-infected cells —●—	6.3×10^5

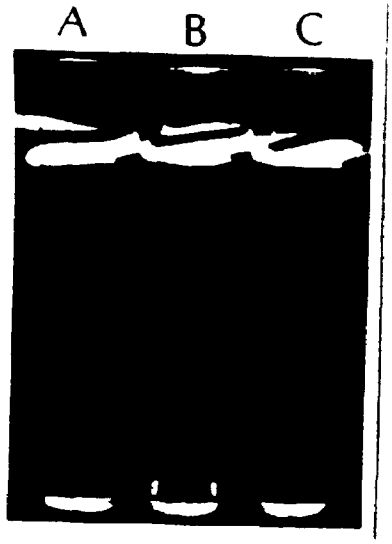
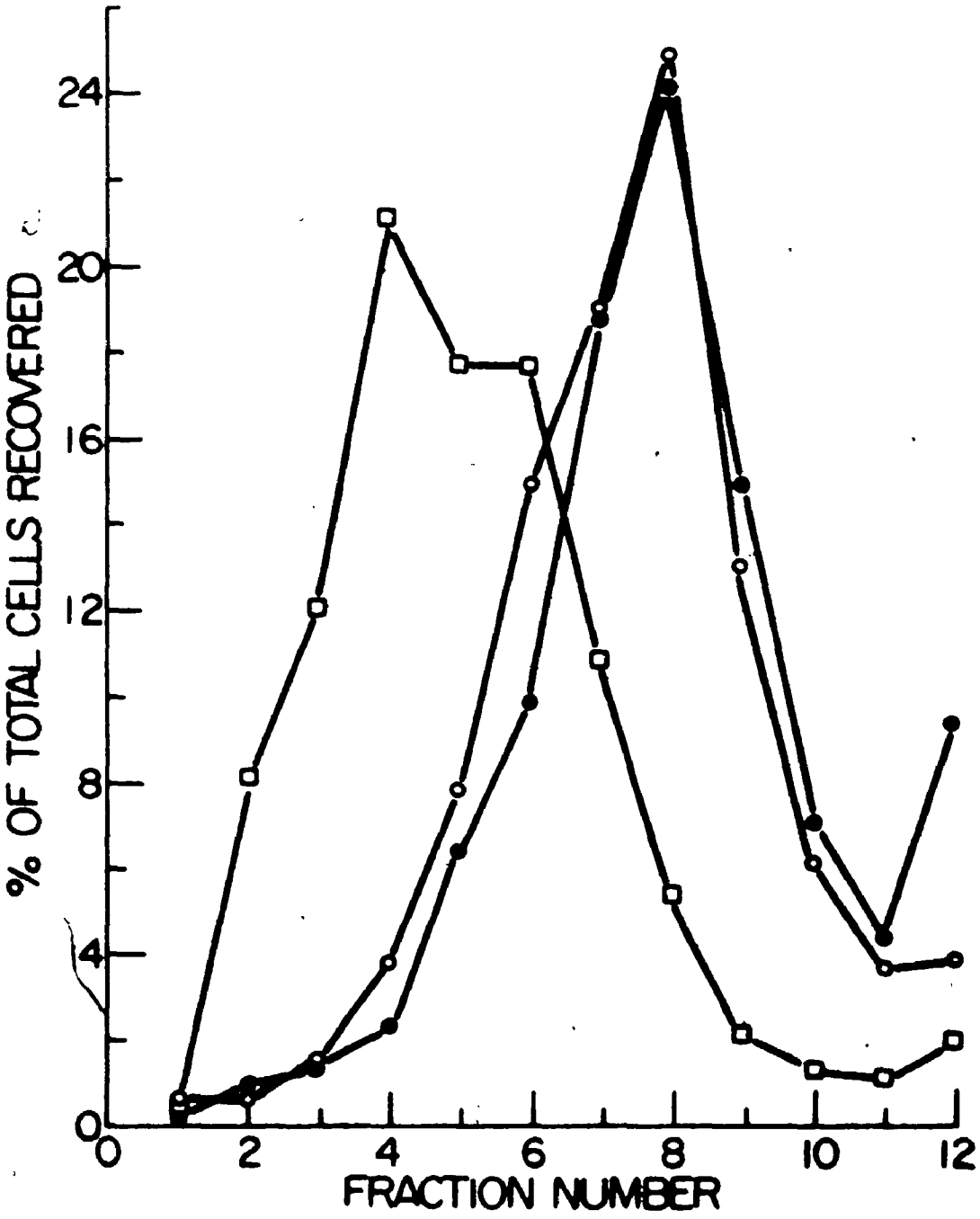


FIGURE 14


% OF TOTAL CELLS RECOVERED
0
4
8
12
16
20
24
0
2
4
6
8
10
12
FRACTION NUMBER

secondary uncoating *per se*, or some event *between* primary and secondary uncoating, is required in order for vaccinia virus to induce the characteristic L-M cell sedimentation distribution change.

K) EFFECT OF HEAT-INACTIVATED VACCINIA VIRUS ON OCCURRENCE OF DISTRIBUTION PROFILE SHIFT PHENOMENON

Another method of inhibiting secondary uncoating, one in which the mechanism is probably a little better understood than U-V irradiation, is by heat-inactivating the input virus. Joklik (1964) found that if vaccinia virus was heated at 60°C for 15 minutes, which reduced the infectivity to below detectable levels (i.e. more than 7 logs in value), secondary uncoating was reduced by 93%, although primary uncoating proceeded normally. Under similar inactivation conditions (56°C for 30 minutes) Dales and Kajioka (1964) determined by electron microscopy that adsorption and penetration had occurred to near normal extents in cells infected with heat-inactivated vaccinia virus. Similar to the conclusions he drew regarding the basis of inactivation by ultra-violet irradiation (see section J), above), Joklik concluded from these observations that heating must have been destroying the "viral inducer protein".

A more up-to-date interpretation of these findings might be that heat inactivation destroyed the virion-bound DNA dependent RNA polymerase. For example, Munyon *et al.*(1970) found that the rate of inactivation of vaccinia virions by heat was reduced in the presence of any one of the four ribonucleotides. Since these ribonucleotides had been shown to act as substrates of the virion-bound enzymes DNA dependent RNA polymerase and nucleotide phosphohydrolase (see Historical Review, section B1), it was suggested that heat treatment was effective in inactivating vaccinia virus by virtue of destroying these virion-bound enzymes.

Assuming that this explanation was correct, it was of interest to determine if these virion-bound enzymes were required to be active in order to produce the typical virus-induced cell sedimentation distribution profile shift. Three cultures of L-M cells were used in this experiment. One culture was mock-infected, one was infected with unheated vaccinia virus, MOI = 10, and one was infected with heat-inactivated vaccinia virus (see Materials and Methods), using an equal volume of the same virus stock to that used in the unheated control. Heat inactivation was found to decrease the titer by virtually 100%; thus the effective MOI of the heated virus-L-M cell system was virtually 0.

Cultures were analyzed at 2 hours PI on standard Ficoll gradients. Results are found in Figure 15. Here it can be seen that heat-inactivated vaccinia virus did not induce the characteristic change in the distribution profile of L-M cells which the unheated virus did. This data therefore suggested that some heat-labile constituent of the vaccinia virion, possibly the virion-bound DNA dependent RNA polymerase, is necessary for the occurrence of the gradient profile shift of vaccinia virus-infected L-M cells.

L) EFFECT OF ANTISERUM-NEUTRALIZED VACCINIA VIRUS ON OCCURRENCE OF DISTRIBUTION PROFILE SHIFT PHENOMENON

A third method of inhibiting secondary uncoating (see sections J) and K) above) is by treating vaccinia virus with virus-specific antiserum prior to infection of cells. Joklik (1964) found that if vaccinia virus was treated with antiserum such that the titer dropped by 92-97%, 75% of the resulting virus-antibody complexes were adsorbed to host cells, relative to untreated control virus. However, 69% of the adsorbed virus failed to penetrate, and was eluted into the medium, as compared to only 16% in the control. The remaining 31% of the adsorbed virus succeeded in penetrating host cells, was uncoated to the core stage, but did not undergo secondary uncoating.

FIGURE 15

EFFECT OF HEAT-INACTIVATED VACCINIA VIRUS ON CELL DISTRIBUTION PROFILE SHIFT AT 2 HOURS PI, AT AN MOI OF 10

Virus was heat-inactivated, cells were infected, and cells were analyzed on Ficoll gradients, as outlined in Materials and Methods.

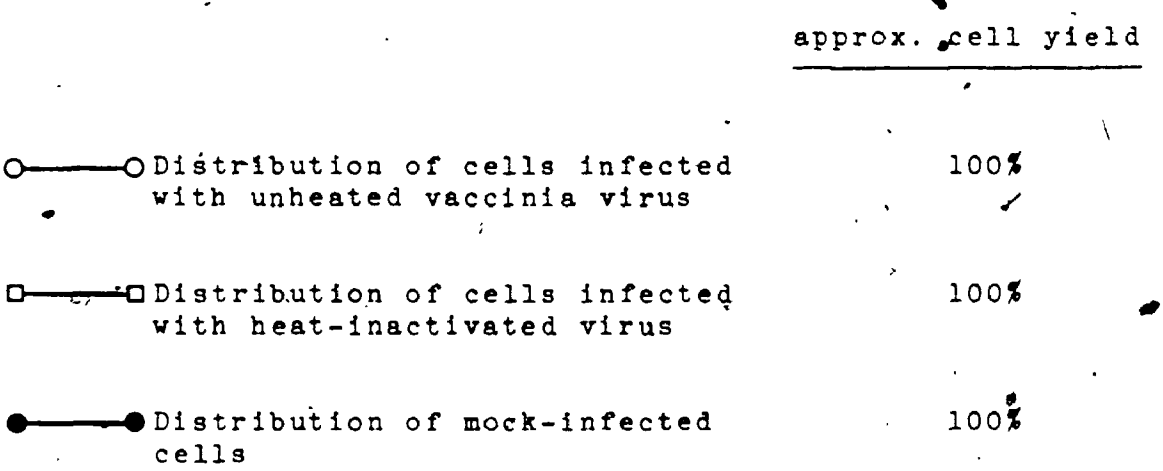
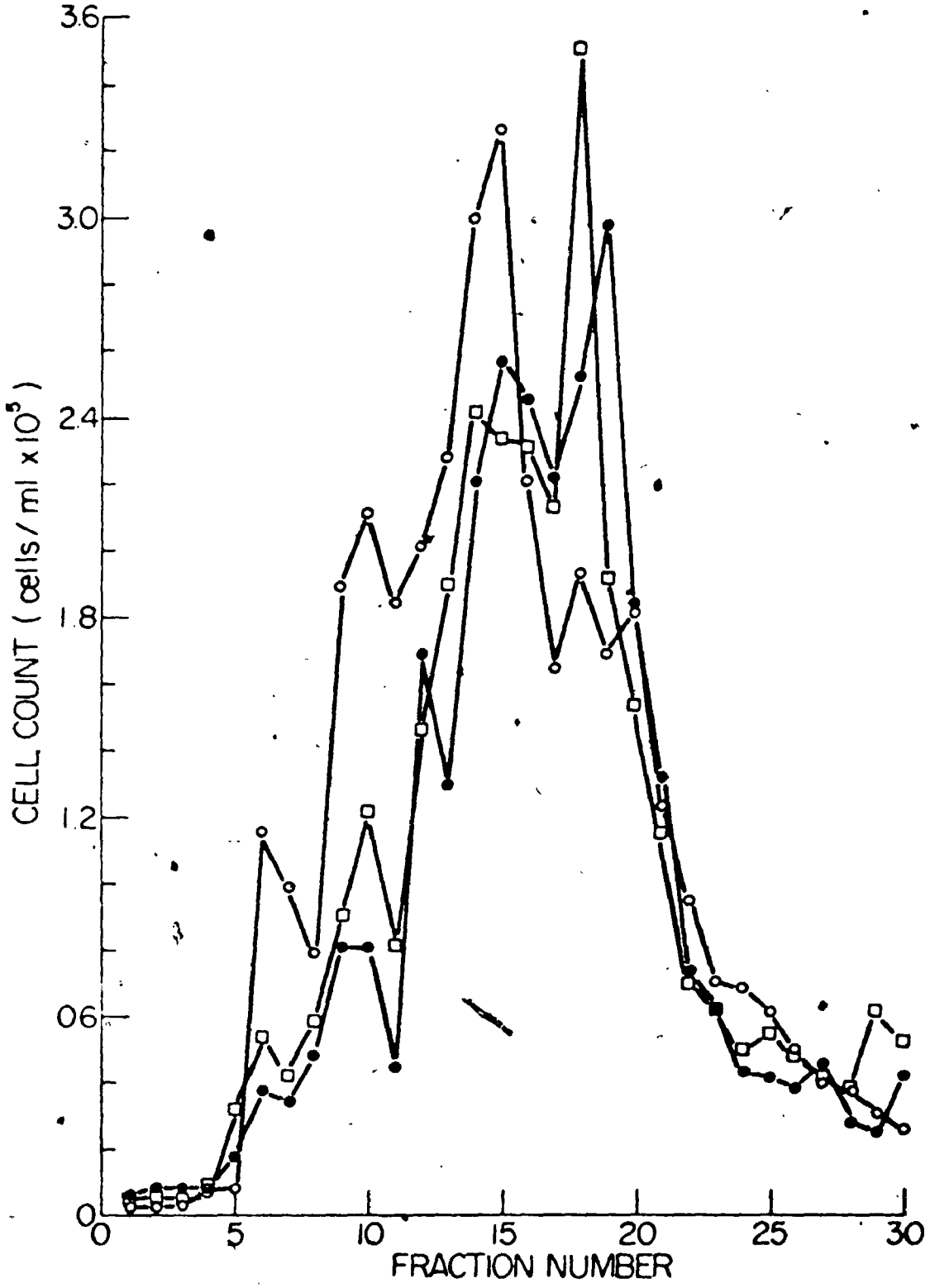


FIGURE 15


3.6
3.0
2.4
1.8
1.2
06
0
CELL COUNT (cells / ml x 10^5)
0
5
10
15
20
25
30
FRACTION NUMBER

Dales and Kajioka (1964) found similar results, except that their data showed that the "majority" (vs. 31%, above) of antibody-treated virus particles were successful in penetrating the host cells.

To possibly gain further insight into the event(s) in the vaccinia virus-infected cell leading to the sedimentation distribution change, an experiment was performed in which L-M cells were infected with antibody-neutralized virus. In this experiment three cultures of L-M cells were used. One culture was mock-infected, one was infected with unneutralized vaccinia virus, MOI = 10, and one was infected with antibody-neutralized vaccinia virus (see Materials and Methods) using an equal volume of the same virus stock to that used in the unneutralized control. Antibody neutralization was found to decrease the titer by 95-99%; thus the effective MOI of the neutralized virus-L-M cell system was at most 0.5.

Cultures were analyzed at 2 hours PI on standard Ficoll gradients. Results are found in Figure 16. Here it can be seen that antibody-neutralized vaccinia virus was able to induce the same characteristic change in the distribution profile of L-M cells that the unneutralized virus induced, in spite of the effective MOI being at most 5% of that of the unneutralized virus control.

This result was unexpected, in view of the results

FIGURE 16

EFFECT OF ANTIBODY-NEUTRALIZED VACCINIA VIRUS ON CELL DISTRIBUTION PROFILE SHIFT AT 2 HOURS PI, AT AN MOI OF 10

Virus was neutralized with specific antiserum, cells were infected, and cells were analyzed on Ficoll gradients, as outlined in Materials and Methods.

		approx. cell yield
A	Distribution of cells infected with antibody-neutralized vaccinia virus —□—□—	94%
B	Distribution of cells infected with unneutralized vaccinia virus —○—○—	100%
C	Distribution of mock-infected cells —●—●—	98%

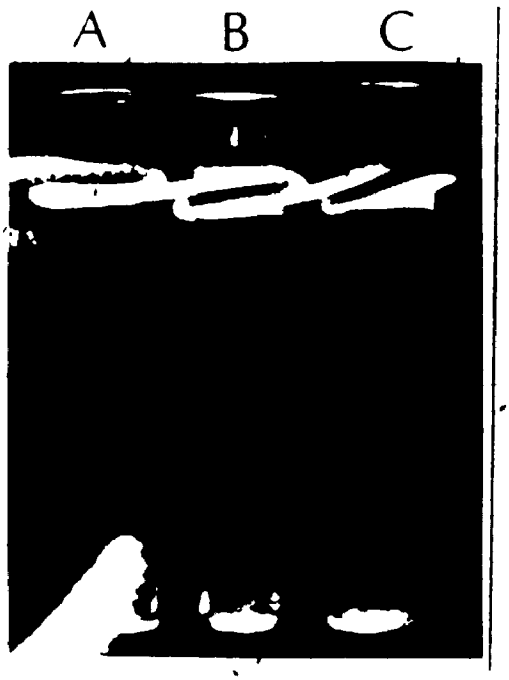
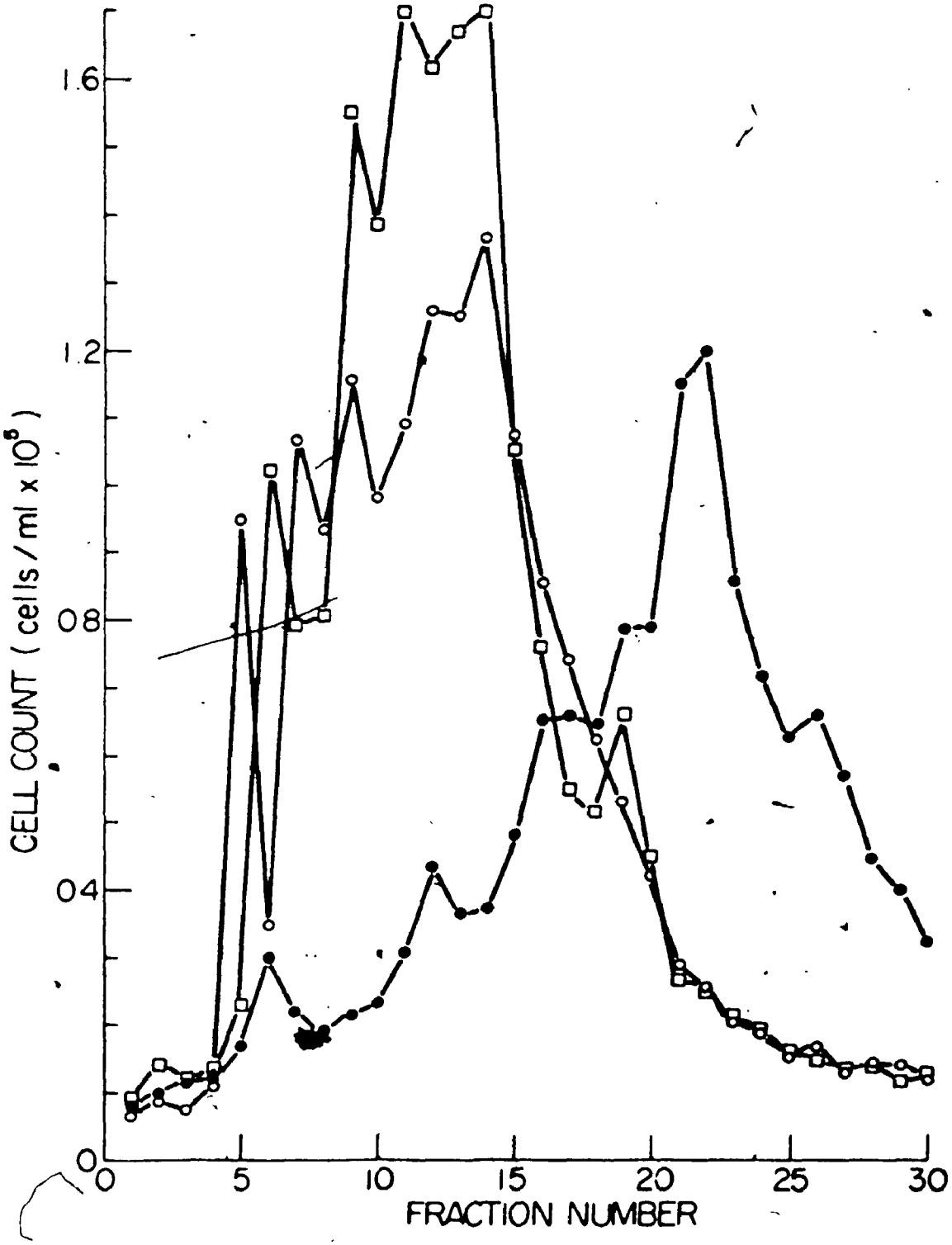


FIGURE 16


1.6
1.2
08
04
0
CELL COUNT (cells / ml x 10^5)
0
5
10
15
20
25
30
FRACTION NUMBER

obtained in sections J) and K) above. Although U-V-inactivated, heat-inactivated, and antiserum-neutralized vaccinia virus apparently all share the same fate in infected cells, namely replicating only to the core state (primary uncoating), they could well differ both in terms of the basis of the inability to undergo secondary uncoating and the manner in which they affect the host cell. This point will be discussed further later in this thesis.

M) EFFECT OF SUBSTITUTING INERT LATEX PARTICLES FOR VACCINIA VIRUS ON THE OCCURRENCE OF THE DISTRIBUTION PROFILE SHIFT PHENOMENON

To rule out the possibility that phagocytosis *per se* might be leading to the distribution profile change described in B) above, cells were subjected to a simulated infection, using uniform polystyrene latex particles, measuring 0.234 μ in diameter, as described in Materials and Methods. This size of particle was almost identical to the dimensions of the vaccinia virion (see Historical Review, section B.1.). Since the physical particle: pfu ratio for vaccinia virus can be as high as 100 (Davis *et al.*, 1973) it was decided to treat L-M cells with both 100 and 1000 latex particles/cell, corresponding to MOI values of 1 and 10 respectively.

Consequently 4 cultures of L-M cells were used in this experiment. One was mock-infected, one was pseudo-infected with 100 latex particles/cell, one was pseudo-infected with 1000 latex particles/cell, and the fourth one was infected with an aliquot of the supernatant (distilled water) (Mr. Lee Bangs, Dow Chemical Co., Midland, Michigan; personal communication) resulting from pelleting the stock latex particles by centrifugation. Viability of all 4 cultures remained $\geq$ 95% during the 2 hr incubation period.

Results at 2 hours PI can be seen in the photograph in Figure 17. From these results it was concluded that L-M cells, treated with as many as 1000 latex particles per cell, do not assume an altered sedimentation distribution pattern when centrifuged on Ficoll gradients.

Assuming that the latex particles adsorbed to and were phagocytized by the L-M cells, it could be concluded that phagocytosis *per se* was not the event taking place within the cell which was leading to the shift phenomenon. (Positive proof of adsorption and phagocytosis of the latex particles, however, would have required either an electron microscopic examination of pseudo-infected L-M cells or the use of radioactively labelled latex particles).

FIGURE 18

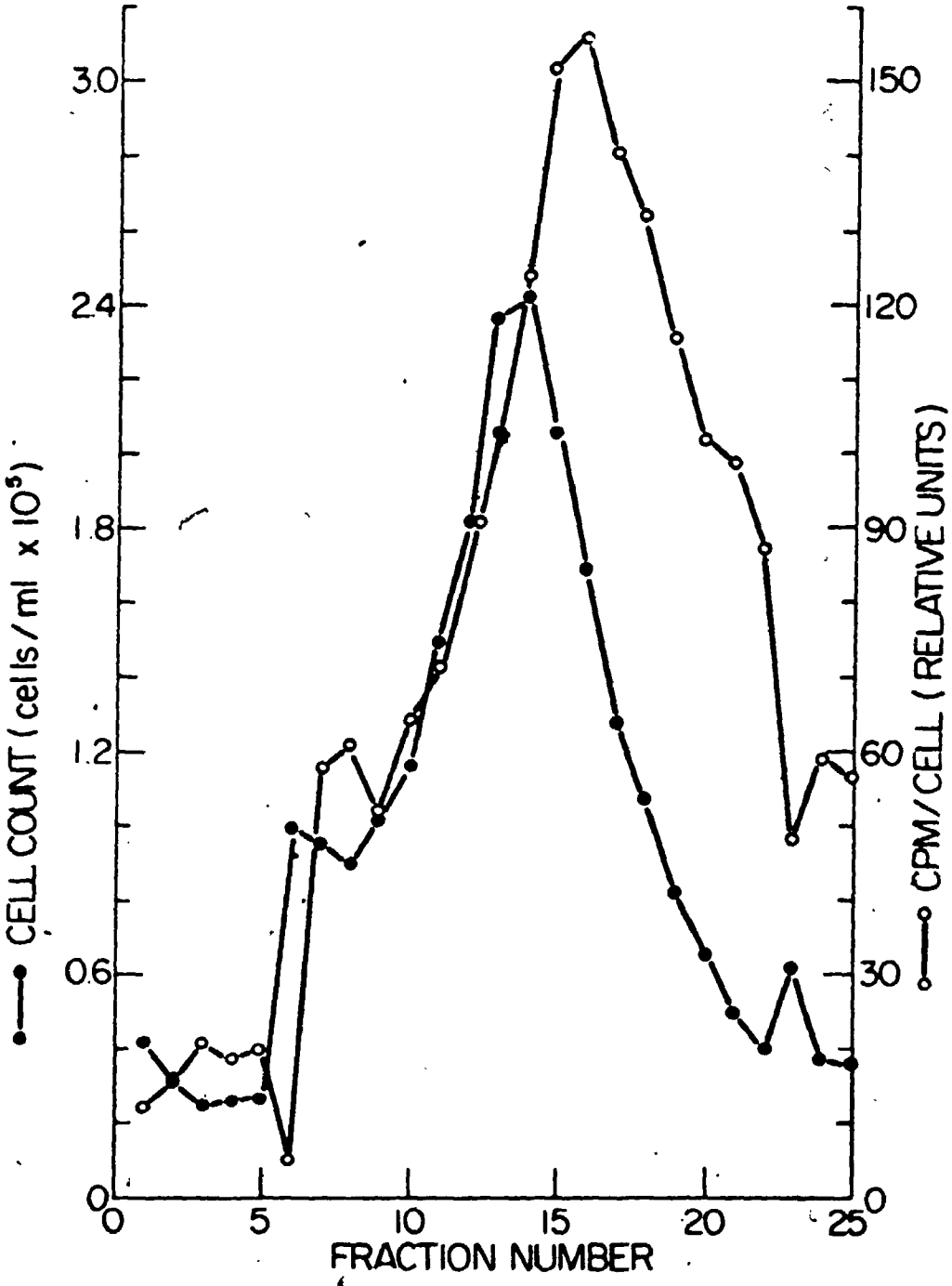
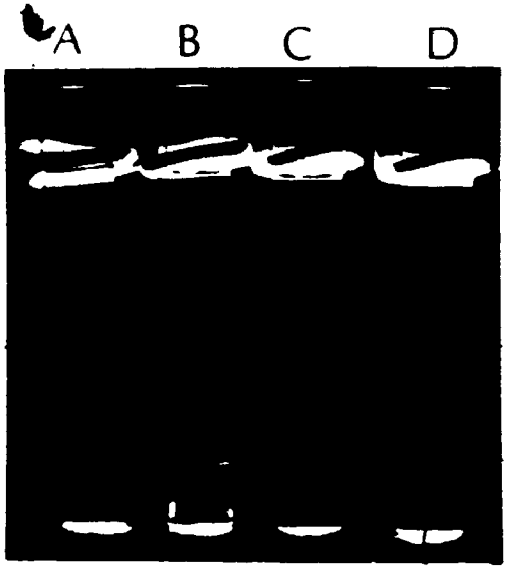

3.0
2.4
1.8
1.2
0.6
0
150
120
90
60
30
0
0
5
10
15
20
25
FRACTION NUMBER
CELL COUNT (cells/ml x 10⁵)
CPM/CELL (RELATIVE UNITS)

FIGURE 17



N) LOCATION OF S PHASE L-M CELLS (FROM A LOG PHASE UNINFECTED POPULATION) IN A FICOLL GRADIENT

The next part of this study was devoted to an examination of the possible relatedness of the distribution profile shift to the eucaryotic cell cycle. A close monitoring of the literature over the past four years has revealed to this investigator a striking increase in the number of studies designed to examine the possible role of the mammalian cell cycle in the replication of various viruses. In many of these studies such a role has been found to exist, including the studies examining vaccinia virus replication (e.g. Groyon and Kniazeff, 1967; Mantani and Kato, 1972).

Since the cell cycle is known to be the basis in many cases for cell separation on density gradients (see Historical Review), it seemed reasonable to investigate the possiblity of the L-M cell cycle being at the root of the phenomenon described in this study. The approach which this investigator chose was to determine whether cells in specific phases of the cycle (e.g. M or S) were specifically contributing to the sedimentation shift.

In order to investigate the possible contribution of S phase cells to the shift phenomenon,

a preliminary experiment had to be performed to determine the location of uninfected S phase cells in Ficoll gradients. A population of log phase uninfected L-M cells was pulsed with ^{3}H-TdR as outlined in Materials and Methods. At the end of the pulse the cells were washed thoroughly to remove all traces of radioactive medium, and were then subjected to centrifugation on 15-22% continuous Ficoll gradients. The gradient was fractionated and each fraction was analyzed for cell concentration and incorporation of ^{3}H-TdR into TCA precipitable counts.

Typical raw data can be found in Appendix 2A. By dividing the cpm incorporated by the number of cells, for each fraction, the value of "cpm/cell" was calculated. This latter term was considered to be an approximate measure of the relative *number* of cells which had incorporated radioactive precursor into TCA precipitable material. The basis for this statement was the close correlation found between "cpm/cell" and the "percent cells showing silver grains" in autoradiography. An example of such a correlation can be found in Appendix 2B, where fractions from a Ficoll gradient were analyzed by Coulter counting and liquid scintillation counting, to arrive at "cpm/cell" values, and by autoradiography for the "percent of cells with

silver grains". The data gave a linear correlation coefficient for these two parameters of 0.78, as computed with the use of a Monroe Epic 3000 programmable printing calculator (Monroe International, Inc., Orange, New Jersey). Thus there existed a very good correlation between the results obtained by liquid scintillation counting (plus Coulter counting) and those obtained by autoradiography. The ease in performing scintillation counting and Coulter counting made this the method of choice over the much more tedious technique of autoradiography.

The data in Appendix 2A is plotted in Figure 18. From these results it was concluded that S phase cells "banded" in a fairly broad zone of the gradient, displaying a fairly typical bell-shaped distribution. The similarity in shape between the "cpm/cell" curve and the "cell count" curve was immediately obvious. The dissimilarity between the two curves lay in the displacement of the peak of the former curve several fractions towards the bottom as compared to the peak of the latter curve. This result was in excellent agreement with those reported by Morris *et al.*,(1967), Warmsley *et al.*,(1969) and others.

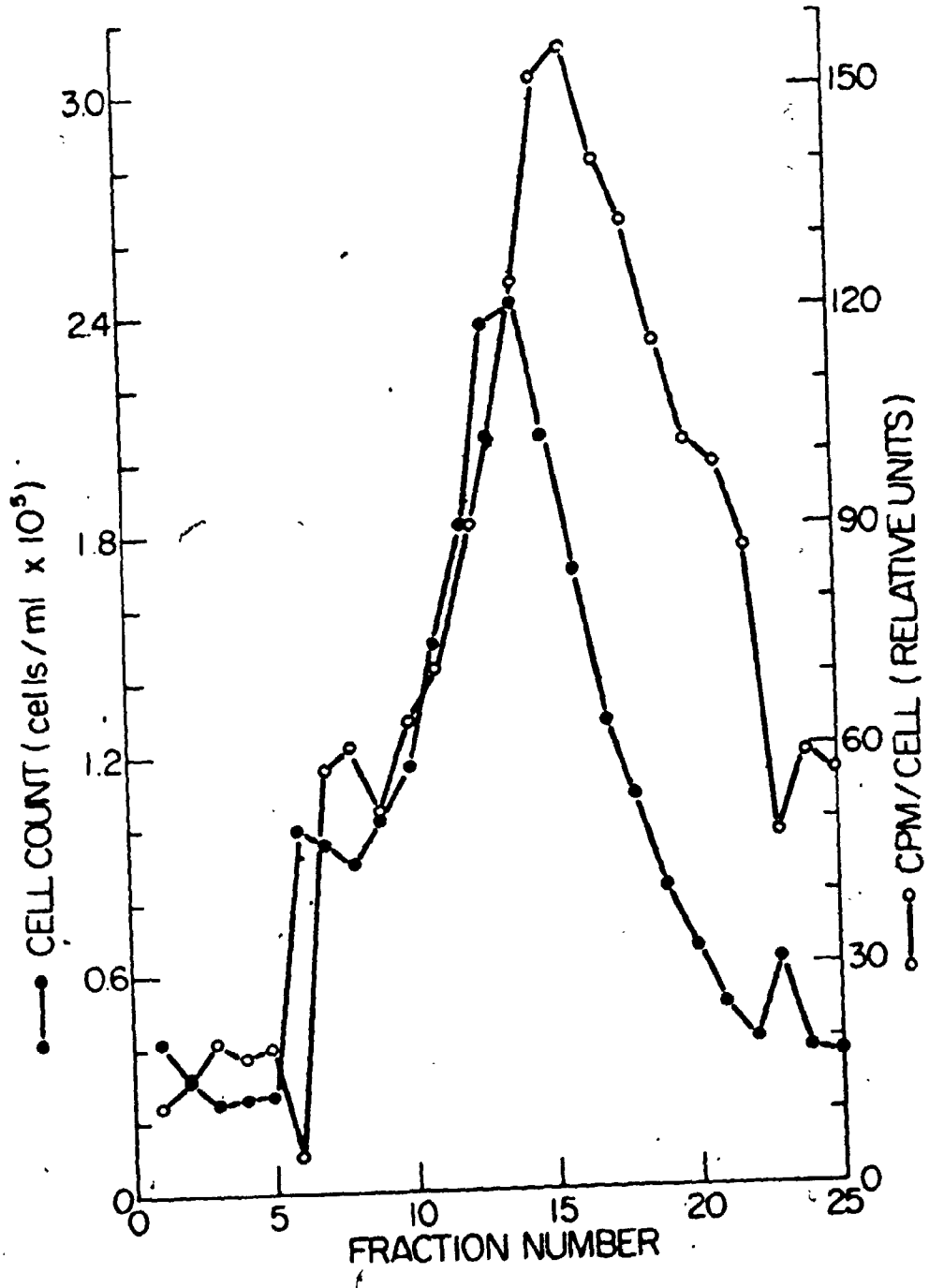
FIGURE 18

LOCATION OF S PHASE CELLS, FROM A LOG PHASE UNINFECTED L-M CELL SUSPENSION CULTURE, IN A FICOLL GRADIENT

Cells were pulsed with ^{3}H-TdR, centrifuged on Ficoll gradients, and fractions were assayed for cell numbers and incorporation of radioactivity, all as described in Materials and Methods. Pulsing time was 1 hr, with 1 µCi/ml radioactive thymidine.

	approx. cell yield
●——● Distribution profile of log phase L-M cells	74%
O——O Distribution profile of S phase cells from a log phase population	

FIGURE 18


3.0
2.4
1.8
1.2
0.6
0
0
5
10
15
20
25
FRACTION NUMBER
CELL COUNT (cells/ml x 10³)
CPM/CELL (RELATIVE UNITS)
150
120
90
60
30
0

O) LOCATION OF LOG PHASE UNINFECTED RADIOACTIVE L-M CELLS IN A FICOLL GRADIENT, WITH TIME AFTER COMPLETION OF A PULSE WITH ^{3}H-TdR

It was argued that if S phase cells were contributing to the distribution profile shift then such a contribution should be demonstrable by virtue of a sedimentation change of these cells and relocation toward the top of a Ficoll gradient. It was already established that under conditions in which every cell received at least one virion (MOI $\geq$ 1), the bulk of cells shifted in their sedimentation position in Ficoll gradients by 2-3 hours PI (Results section D). Although this finding tended to suggest that all cells (i.e. cells in all stages of the cell cycle) might be contributing to the shift, it was noted that there was always a small but significant number of cells which did not relocate in the gradient. Thus the possibility did seem to exist that only certain cells, from the general infected cell population, were being specifically recruited into forming a population with altered sedimentation properties. One certain type of cell which was relatively easy to differentiate from the entire log phase population was the S phase cell, by virtue of the fact that only cells in S phase can incorporate radioactive thymidine into TCA precipitable material.

Before determining if cells in S phase at the time of infection were contributing to the shift, however, it was necessary to see whether *uninfected* radioactive cells ever occupied a "shifted" position in a Ficoll gradient. Since it had already been established that radioactive cells immediately after a pulse with ^{3}H-TdR located in a position slightly below the main cell peak (see N above), and that M phase cells banded close to the top of identical Ficoll gradients (see Q below), it seemed not unreasonable to suspect that if cells in S phase proceeded into G2 and M, such "marked" cells ("marked" by virtue of being radioactive) might possibly band somewhere in the gradient *between* the top and a position slightly below the main cell peak.

The results of such a preliminary experiment can be found in Figure 19. As can be seen from this figure, a 1-2 fraction shift in the distribution profile of the entire cell population occurred between 0 and 4 hours post pulse. This shift was unexpected and did not occur at 2.5 hours (results not shown). An experiment was carried out to determine if this unexpected shift might have been due to the excess thymidine introduced into the medium as ^{3}H-TdR. Results (not shown) indicated that addition of an equal amount of unlabelled TdR to that present in the pulse did not lead

FIGURE 19

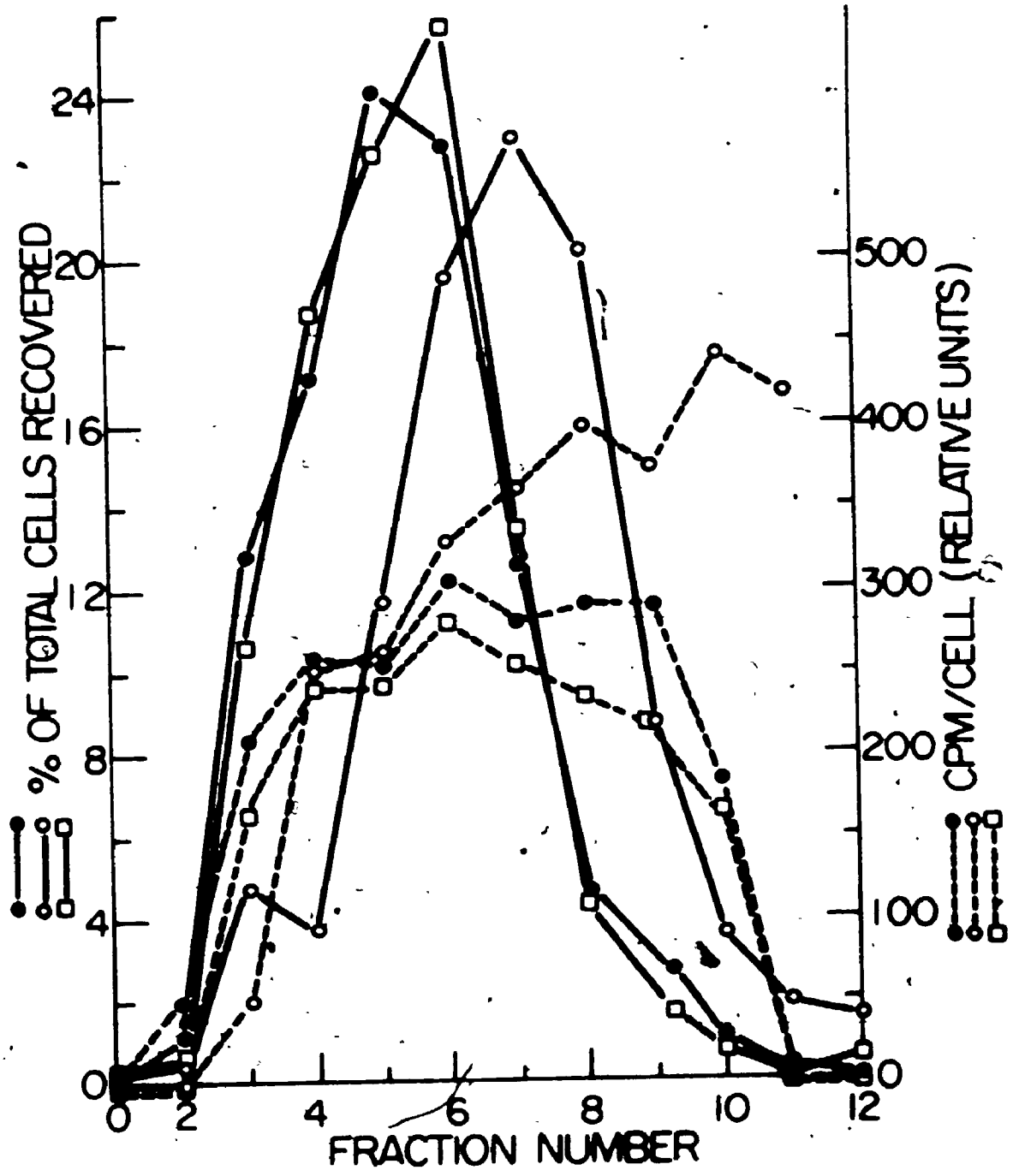
LOCATION OF ^{3}H-TdR-PULSED UNINFECTED LOG PHASE SUSPENSION CULTURE L-M CELLS IN A FICOLL GRADIENT, AT VARIOUS TIMES DURING THE CHASE

A log phase L-M cell population was pulsed with ^{3}H-TdR, centrifuged on Ficoll gradients, and gradient fractions were assayed for cell numbers and incorporation of radioactivity, all as described in Materials and Methods. Cell load for each gradient = 7.6×10^{5}.

	Total number of cells recovered
○——○ Cell distribution profile of the entire population, 0 hours PP*	4.8×10^{5}
●——● Cell distribution profile of the entire population, 4 hours PP	7.5×10^{5}
□——□ Cell distribution profile of the entire population, 16 hours PP	5.6×10^{5}
○- - -○ Distribution profile of radioactive cells, 0 hours PP	
●- - -● Distribution profile of radioactive cells, 4 hours PP	
□- - -□ Distribution profile of radioactive cells, 16 hours PP	

*PP - post pulse

FIGURE 19


% OF TOTAL CELLS RECOVERED
CPM/CELL (RELATIVE UNITS)
FRACTION NUMBER
0
4
8
12
16
20
24
100
200
300
400
500
0
2
4
6
8
10
12

to any cell distribution profile shift. Thus the nature of this anomaly was not explained.

From Figure 19 it was noted that none of the three "cpm/cell" curves showed any obvious peaks such as were found on the other three curves in this figure. Thus it appeared that no restricted region of the gradient was highly enriched in radioactive cells. This finding prompted this investigator to use the following argument: Even if the cell separation technique being used in this study seems to be incapable of always producing a fraction highly enriched with radioactive cells (contrast with the data of Figure 18), thereby making it impossible to detect a repositioning of such a fraction of cells with time post pulse, it might be possible to detect a *trend* of radioactive cells to reposition themselves in a Ficoll gradient. Such a trend should be detectable if a difference could be shown in the 'overall position' of radioactive cells in a gradient.

It was decided that an 'overall position' of radioactive cells could best be expressed quantitatively as a slope of the linear regression curve (best straight line) of the "cpm/cell" vs."fraction number" data. For example, if radioactive cells assume an overall position near the bottom of a standard gradient at 0 hours post pulse, it would be predicted that the slope of the linear

regression curve would be positive in value. If with time post pulse these radioactive cells began to assume positions nearer the top of the gradient, it would be predicted that the slope of the linear regression curve would decrease in value, perhaps even to a negative value, whereas if these radioactive cells tended to remain in the same position in the gradient, then the slope of the linear regression curve would remain unchanged.

The "cpm/cell" and "fraction number" data from Figure 19 was used to compute 3 linear regression curves, for 0, 4, and 16 hours post pulse. In each case only the data for the "cpm/cell" curve which fell within the corresponding "percent of total cells recovered" curve was used for the computation. The data was treated as exemplified in Appendix 3A. The slopes of the linear regression curves were computed from the treated data, with the use of the Monroe Epic 3000 calculator, and were found to have values of 46, 19, and 22 for the 0 hour, 4 hour, and 16 hour post pulse samples, respectively.

An analysis was then performed on these slope values to see if any two values differed significantly at the 95% confidence level. An example of such a calculation, for the slopes of the 0 hour and 4 hour post pulse linear regression curves from Figure 19, is outlined in Appendix 3B. From such calculations it was found that the 4 hour post pulse linear regression curve

slope did not differ significantly from the 0 hours post pulse linear regression curve slope, and that the 16 hours post pulse linear regression curve slope also did not differ significantly from the 0 hours post pulse linear regression curve slope. The conclusion drawn from these findings was that, in an uninfected L-M suspension culture, cells which incorporated ^{3}H-TdR during a 1 hour pulse did not assume an altered sedimentation property (decreased density?) in Ficoll gradients, when analyzed for 16 hours into the chase.

P) LOCATION OF VACCINIA VIRUS-INFECTED RADIOACTIVE L-M CELLS IN A FICOLL GRADIENT, WITH TIME AFTER COMPLETION OF A PULSE WITH ^{3}H-TdR

From the results obtained in O) above it was clear that S phase cells in an uninfected log phase population do not change in their sedimentation behaviour during 16 hours of chase in cold (non-radioactive) medium.

Using the same approach as that taken in O), the question was then asked whether cells in S phase at the time of infection with vaccinia virus were contributing to the distribution shift. If they were, then the slopes of the linear regression curves of the "cpm/cell" vs. "fraction number" data should decrease with time into the chase. If they were not contributing, then the slopes should remain relatively constant.

An experiment was performed to determine which of these possibilities the data supported. In this experiment 2 cultures of L-M cells were employed, one mock-infected and one infected with vaccinia virus, MOI=1. Cells were pulsed with ^{3}H-TdR for one hour, were washed well to remove unincorporated material, and then were infected with vaccinia virus. This time was considered as 0 hours post pulse, as well as 0 hours PI. At 0, 3, and 8 hours PI an aliquot from each culture was analyzed on a standard Ficoll gradient.

Results are seen in Figure 20. At 0 hours PI the 2 cell distribution curves were almost coincident; by 3 hours PI there was a slight shift in the distribution profile of the infected culture toward the top of the gradient, and by 8 hours PI the cells of the 2 populations were quite well separated in the gradient. Linear regression curve calculations of the "cpm/cell" values were performed on the 0, 3, and 8 hour data, as outlined in Appendix 3A. Slopes of these best straight lines were found to be 47, 23, and 4, respectively.

Using the formula outlined in Appendix 3B it was found that the decrease in the slope value from 47 to 23, occurring between 0 hours PI and 3 hours PI, was not statistically significant at the 95% confidence level ($t_{calc} = 1.58$; $t_{.05} = 1.77$). However, the decrease

FIGURE 20

LOCATION OF ^{3}H-TdR-PULSED VIRUS-INFECTED SUSPENSION CULTURE L-M CELLS IN A FICOLL GRADIENT, AT VARIOUS TIMES DURING THE CHASE

A log phase cell population was pulsed with ^{3}H-TdR, infected with vaccinia virus at an MOI of 1, centrifuged on Ficoll gradients, and gradient fractions assayed for cell numbers and amount of incorporated radioactivity, all as described in Materials and Methods. Cell loads were: (A) 8.8×10^5; (B) 10.3×10^5; (C) 8.8×10^5.


Cell distribution profile of mock-infected cells


Cell distribution profile of virus-infected cells


Calculated distribution profile of radioactive cells in the virus-infected culture

TOTAL NUMBER OF CELLS RECOVERED

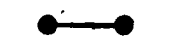
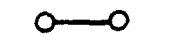
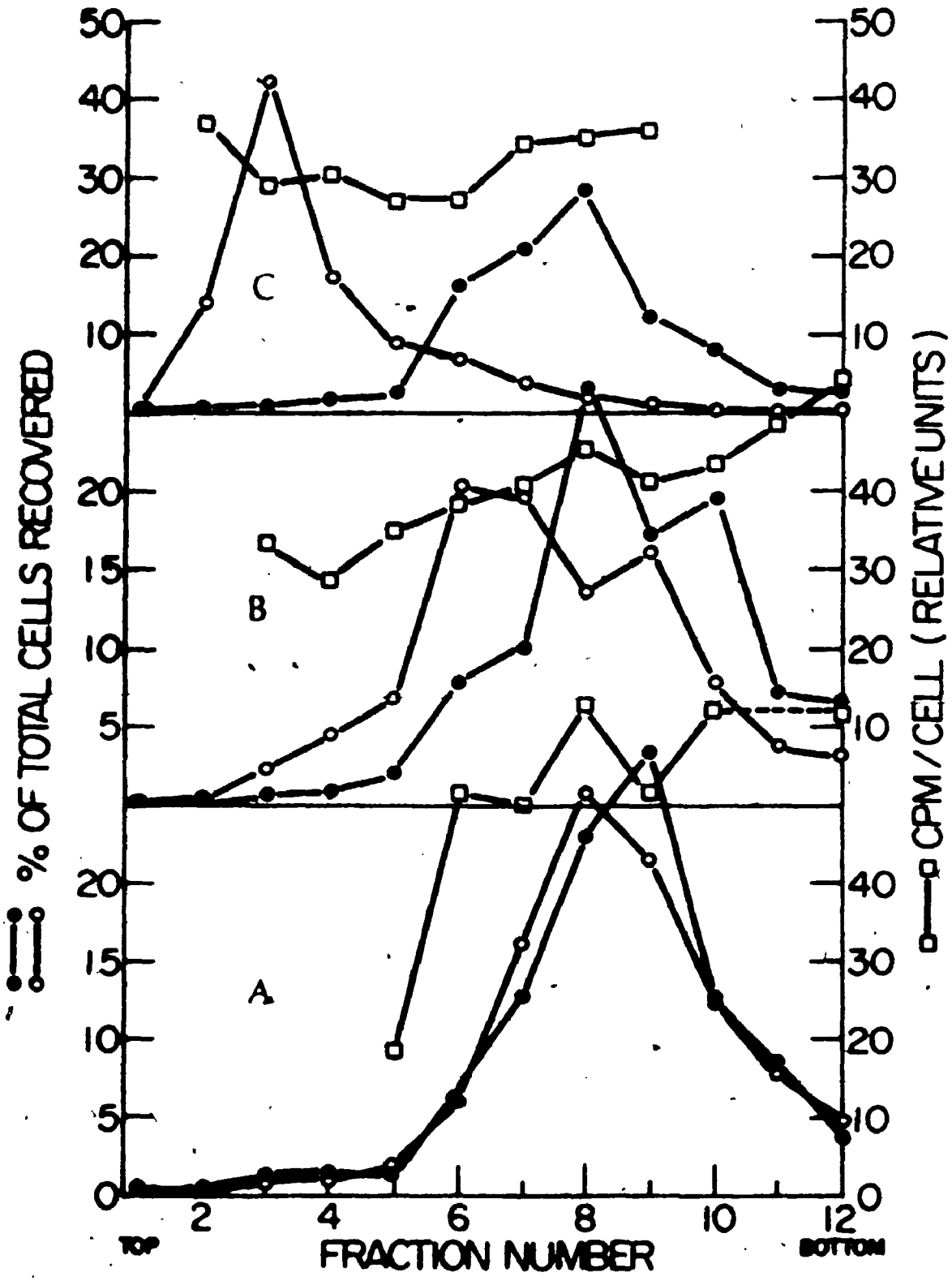
			
A	0 hour PI	4.3×10^5	5.0×10^5
B	3 hours PI	6.3×10^5	7.2×10^5
C	8 hours PI	5.7×10^5	7.8×10^5

FIGURE 20


% OF TOTAL CELLS RECOVERED
CPM/CELL (RELATIVE UNITS)
FRACTION NUMBER
TOP
BOTTOM
A
B
C

in slope value from 23 to 4, occurring between 3 hours PI and 8 hours PI, was definitely significant at the 95% confidence level ($t_{calc} = 3.1$; $t_{.05} = 1.76$).

The conclusion drawn from these results was that cells in S phase at the time of infection were definitely being recruited into a population of cells which exhibited an altered sedimentation property in a Ficoll gradient, and in this way were contributing to the phenomenon. The fact that this contribution was not evident by 3 hours PI should not be considered disturbing in view of the fact that with the low MOI used in this experiment the shift phenomenon only becomes apparent after 3 hours PI, as found in Figure 20.

Q) LOCATION OF M PHASE L-M CELLS (FROM A LOG PHASE UNINFECTED POPULATION) IN A FICOLL GRADIENT

Using a similar relationale to that used for S phase cells (see N) above), it was decided to determine the possible contribution of M phase cells to the shift phenomenon. Several preliminary experiments were performed to determine the location of M phase cells from uninfected populations on Ficoll gradients. These preliminary experiments differed from each other only in the method by which M phase cells were obtained.

(i) M PHASE CELLS OCCURRING NATURALLY WITHIN A LOG PHASE SUSPENSION CULTURE POPULATION

An aliquot from a normal log phase asynchronous culture of L-M cells was chilled briefly and centrifuged on a 15-22% continuous Ficoll gradient, following the standard procedure. The gradient was fractionated and each fraction was analyzed for cell count and percent cells in mitosis (mitotic index; see Materials and Methods). In every case the unfractionated cell population had a mitotic index of 2-3% i.e. 2-3% of all cells examined (usually 500) showed distinct chromosomes and were scored as positive by virtue of this property. Typical results can be found in Figure 21. Clearly it was seen that the peak of cells in mitosis banded at the top of the gradient, usually at the interface between the sample volume and the Ficoll. This peak contained approximately a 10-fold enrichment of M phase cells as compared to the general population (e.g. 32% in one case, 25% in another). Since the uppermost fractions contained so few cells, it was often impossible to score 500 cells. Thus the error for the mitotic index was greater in the upper fractions. In no case, however, were less than 50 cells scored.

(ii) M PHASE CELLS INDUCED BY COLCEMID

Two cultures were used for this experiment. One

FIGURE 21

LOCATION OF NATURALLY-OCCURRING M PHASE CELLS, FROM A LOG PHASE UNINFECTED CELL SUSPENSION CULTURE, IN A FICOLL GRADIENT

Cells were centrifuged as described in Materials and Methods. Fractions from the gradient were analyzed for cell numbers and cells possessing mitotic figures, as outlined in Materials and Methods.

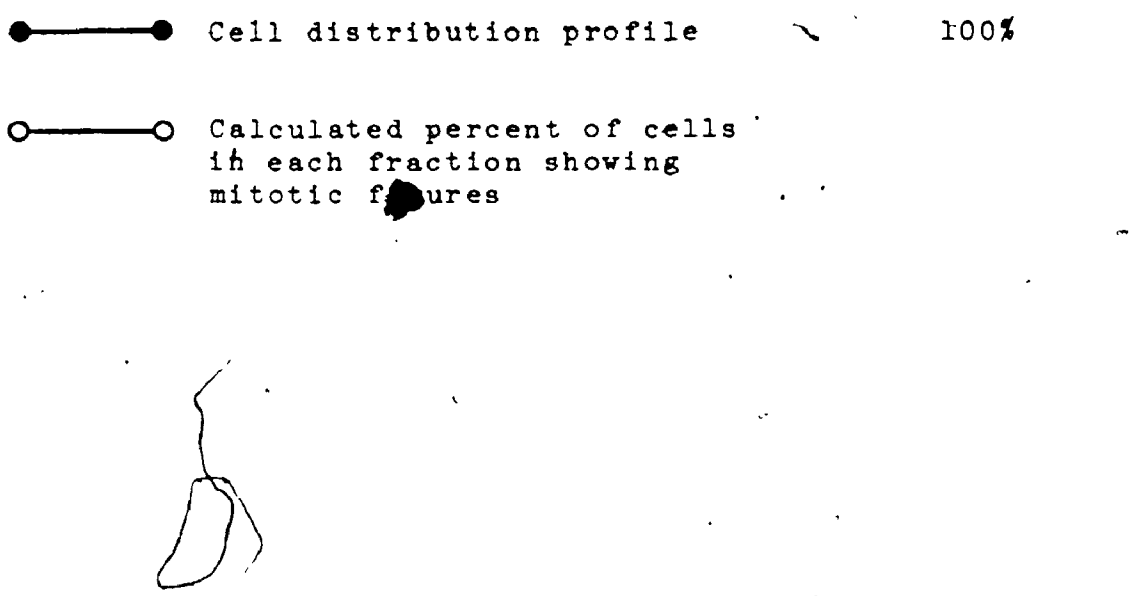
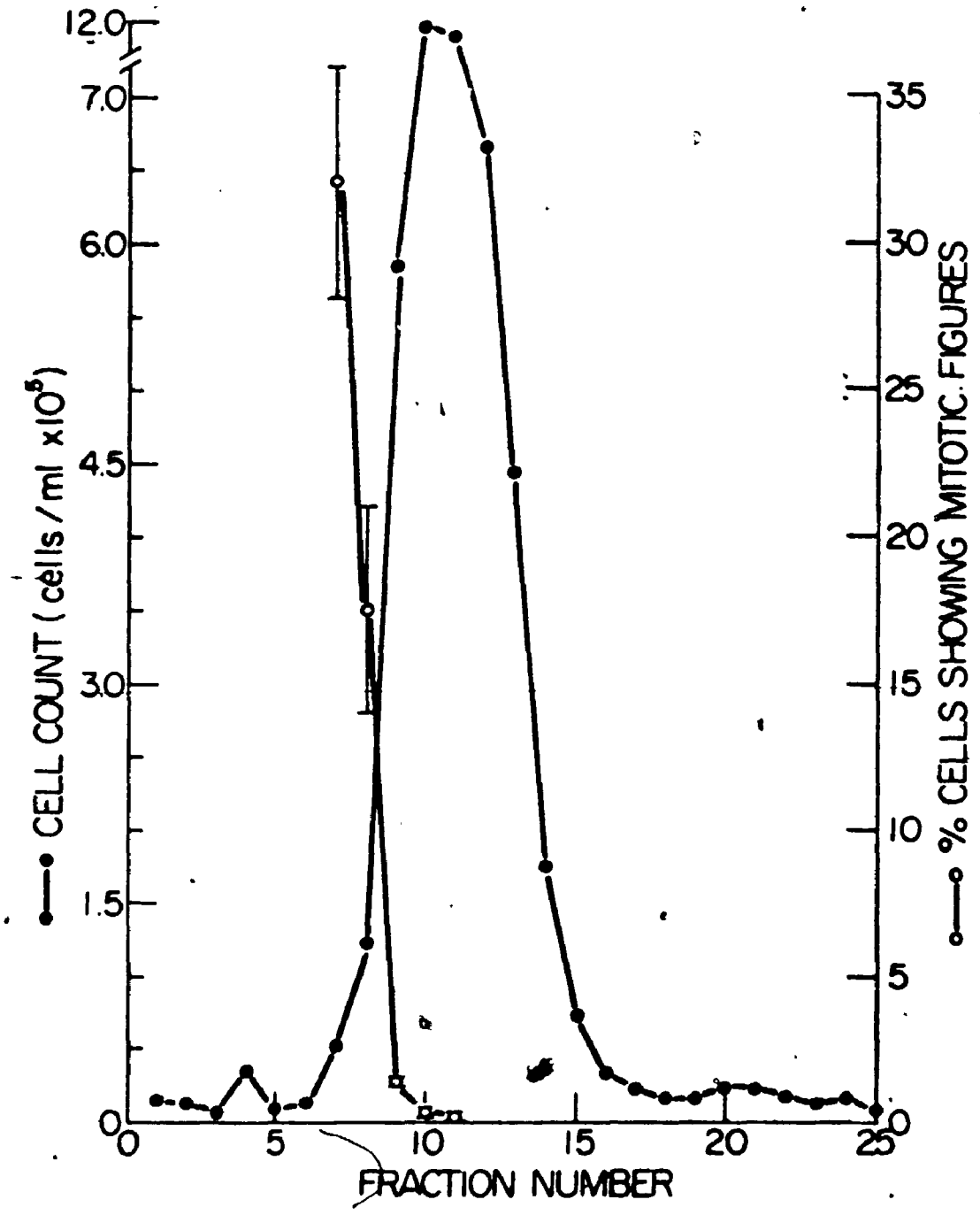


FIGURE 21


12.0
7.0
6.0
4.5
3.0
1.5
0
CELL COUNT (cells/ml x10⁵)
35
30
25
20
15
10
5
0
% CELLS SHOWING MITOTIC FIGURES
0
5
10
15
20
25
FRACTION NUMBER

culture was incubated with colcemid, as described in Materials and Methods, and the other with medium 199P+MC in place of colcemid. After 24 hours incubation the mitotic index of the drug-treated culture was found to be 20%, whereas the control culture was 1.3%. By 40 hours this value had increased to 27-45%, depending on the experiment. After colcemid treatment for 24 hours cells were analyzed for their distribution in a standard Ficoll gradient. Results (not included) showed no major difference in the distribution profile, with the possible exception of a slight increase in cells at the top of the gradient, in the case of the drug-treated cells. By 72 hours a major change had taken place. Now the drug-treated cells exhibited a distribution profile markedly different from the control cell distribution profile, as can be seen in Figure 22.

An aliquot of a 40 hour drug-treated culture was analyzed for cell distribution in a standard Ficoll gradient and for mitotic index. Results were similar to those found in Figure 21 for normally occurring M cells. The peak of M cells occurred at the top of the gradient, where 83% of the cells exhibited mitotic figures, whereas the corresponding fraction in the "naturally occurring" population had a mitotic index of only 32% (see Figure 21). Since the 40 hour drug-treated unfractionated culture

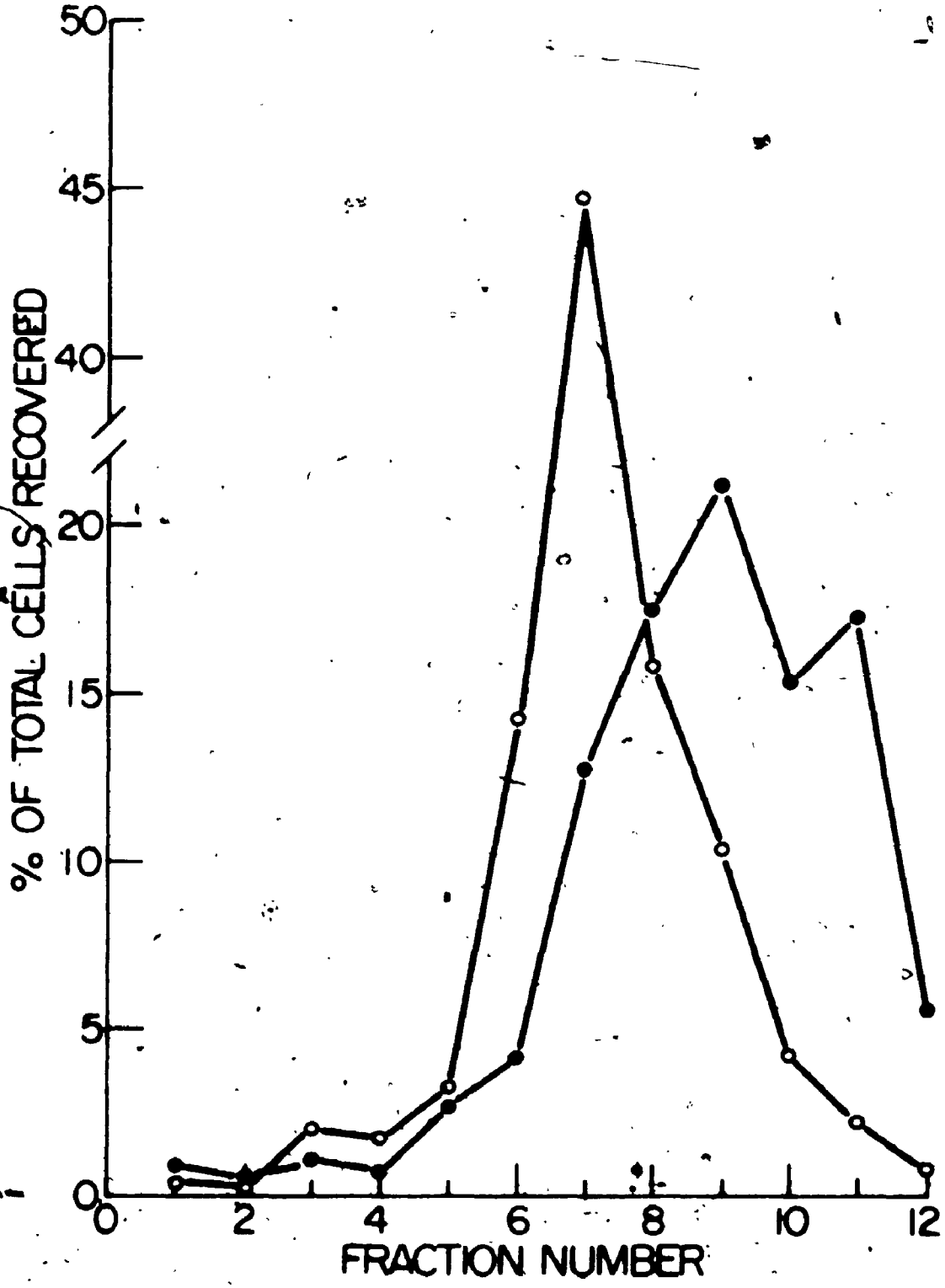
FIGURE 22

SEDIMENTATION DISTRIBUTION IN A FICOLL GRADIENT OF 72 HOUR COLCEMID-TREATED SUSPENSION CULTURE L-M CELLS

Cells were treated with colcemid and centrifuged on a Ficoll gradient as described in Materials and Methods. Cell loads for each gradient = 8.8×10^5.

		Total number of cells recovered
●——●	Cell distribution profile of untreated cells	3.8×10^5
○——○	Cell distribution profile of 72 hour colcemid-treated cells	3.5×10^5

FIGURE 22


50
45
40
20
15
10
5
0
% OF TOTAL CELLS RECOVERED
0
2
4
6
8
10
12
FRACTION NUMBER

exhibited a mitotic index of 45%, the enrichment obtained in the top-most fraction (from 45% to 83% i.e. less than 2-fold) of the drug-treated culture was not nearly as great as the enrichment obtained in the top-most fraction (from 2-3% to 32% i.e. approximately 10-fold) of an untreated culture, although the preponderance of mitotic cells found in the top-most fraction was greater as a result of treatment with colcemid (83% vs. 32%).

The general conclusion reached from this experiment was the same as that in Q) (i) above; namely that M phase cells, in this case induced by the cell cycle arresting action of colcemid, banded toward the top of standard Ficoll gradients.

(iii) M PHASE CELLS OBTAINED FROM ROLLER BOTTLES

The shake-off contents of 3 confluent roller bottles, obtained using the apparatus depicted in Figure 23, were pooled, and analyzed on standard Ficoll gradients. Results can be found in Figure 24. Individual fractions were not analyzed for mitotic indexes. The entire unfractionated population had a mitotic index of 40-50%. In comparing these results with those found in (ii) above, it appeared that shake-off cells were displaced towards the top of the gradient further 4

FIGURE 23

ROLLER BOTTLE APPARATUS OF SANDERS, MEDZON, AND BALL

A. Base and pedestal

B. Roller drum sides

C. Roller drum spacers

D. Main bearing

E. Shaft to bearing coupling

F. Roller drum hub

G. Roller drum shaft

H. Drive assembly hinge

K. Drive assembly pulley

L. Drive assembly bearing housing

N. Drive assembly drive shaft

O. Drive assembly motor

P. Drive assembly drive belt

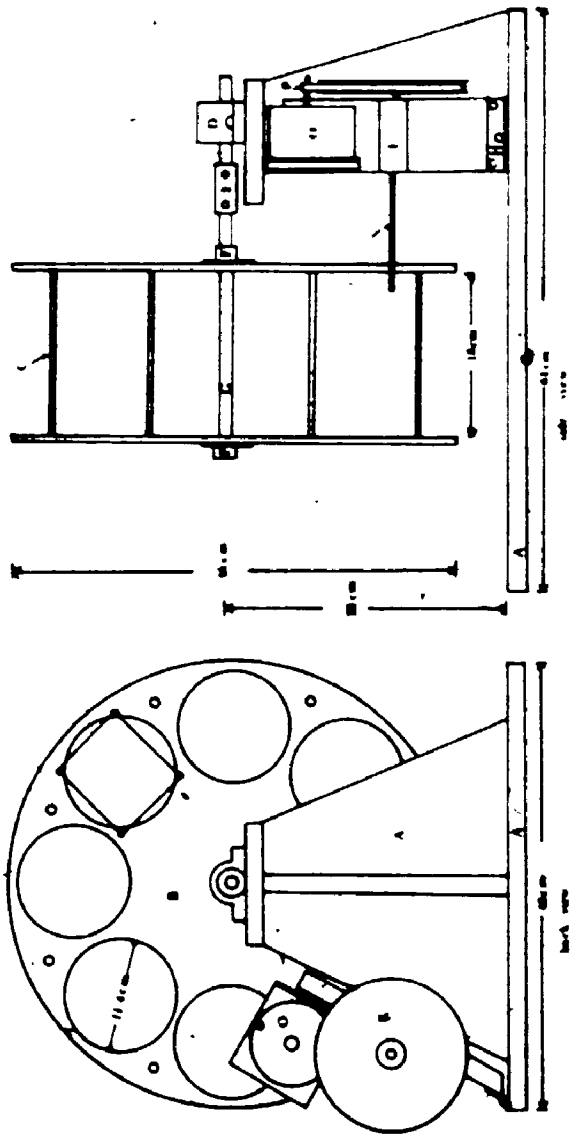


FIGURE 23

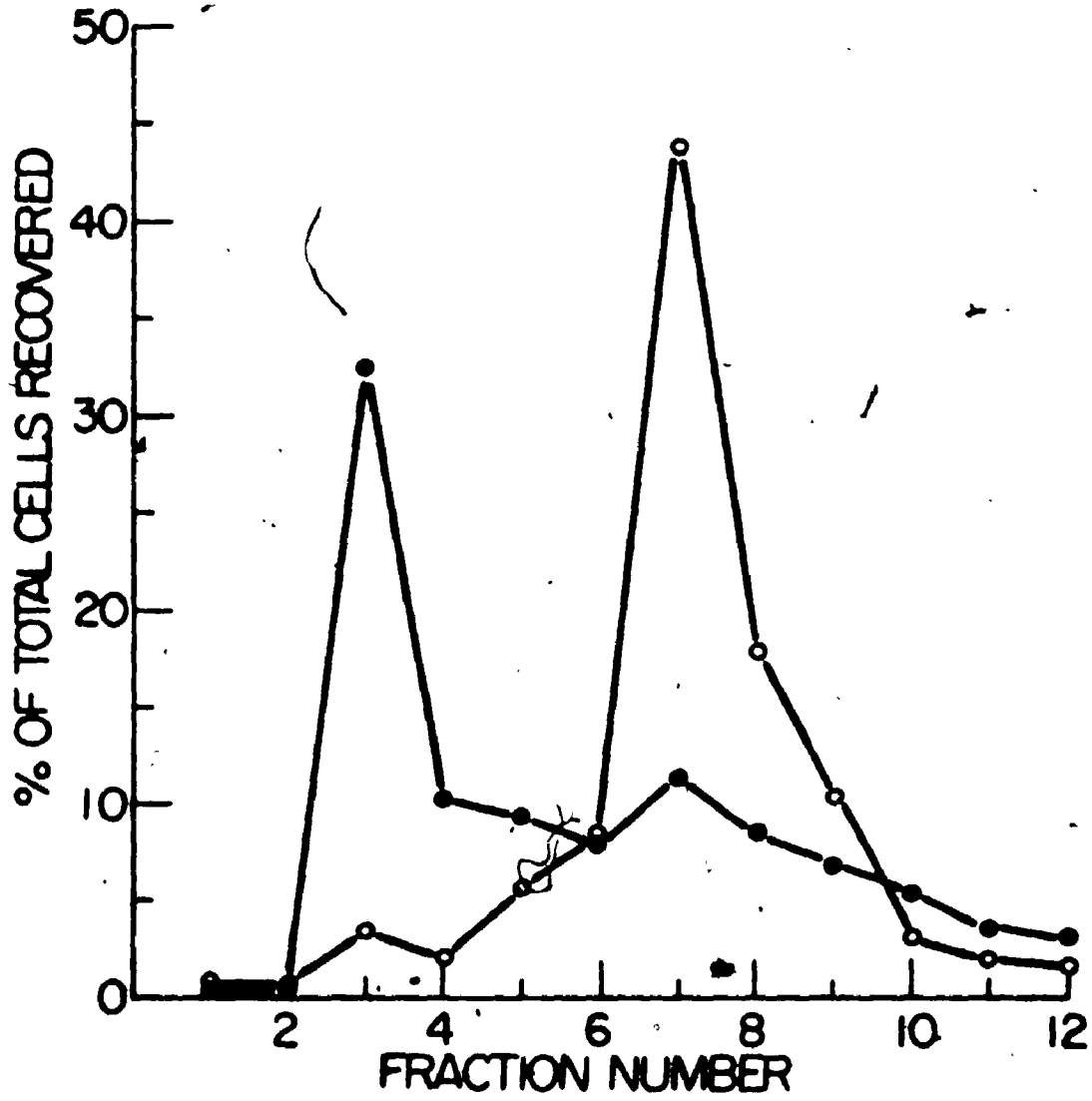
FIGURE 25

LOCATION OF M PHASE VACCINIA VIRUS-INFECTED SUSPENSION CULTURE L-M CELLS IN A FICOLL GRADIENT, AT 3 HOURS PI AT AN MOI OF 1

Conditions for infection and analysis were as described in Materials and Methods. Cell yields were: 97% (virus-infected); 45% (mock-infected).

		Total number of cells recovered
○——●	Cell distribution profile of virus-infected cells, and	4.0×10^5
○- - -○	the percent of these cells showing mitotic figures in each fraction.	
●——●	Cell distribution profile of mock-infected cells, and	8.5×10^5
●- - -●	the percent of these cells showing mitotic figures in each fraction.	

FIGURE 24


50
40
30
20
10
0
% OF TOTAL CELLS RECOVERED
2
4
6
8
10
12
FRACTION NUMBER

fractions) from their controls than were the colcemid-treated cells (2 fractions) from their controls. This finding is very similar to that reported by Wolff & Pertoft (1972), who noted that HeLa cells blocked in metaphase by colcemid were lighter in density than interphase cells, but somewhat more dense than untreated cells selected by density gradient centrifugation.

In general it could be stated that M cells naturally occurring, obtained by cell cycle inhibition with colcemid, or collected in large amounts by shake-off, banded near the top of standard Ficoll gradients, well above the position occupied by the majority of L-M suspension culture cells.

R) LOCATION OF M PHASE VACCINIA VIRUS-INFECTED L-M CELLS IN A FICOLL GRADIENT, AT 3 HOURS PI

Following the rationale used for S phase cells (see O above), the next step should have been an investigation of the location of cells, which were in M phase at zero time, as incubation of an uninfected population proceeded. For two reasons, however, this approach was not pursued: First because, unlike S phase cells, there is no convenient way to "mark" M cells in order to follow them after they leave the mitotic phase of the cell cycle;

and secondly because, even if a way did exist to "mark" M phase cells, the results of experiments with *S phase cells* suggested that no significant change in position of these cells in a Ficoll gradient would occur for many hours after "marking" the cells. (Since the M phase takes cells only a very short time to traverse, relative to the S phase (see section S), the application of this second argument to the M cell situation may not be valid, however).

Instead, the line of investigation taken was simply to examine the location of cells in M phase in a Ficoll gradient at 3 hours after infection of a log phase population with vaccinia virus. The short time after infection (3 hours) was chosen for analysis in order to minimize the chance of M cells at 0 hours PI moving away from their characteristic sedimentation position near the top of Ficoll gradients, thereby confusing the interpretation of the results.

From section Q) it was known that naturally occurring M phase cells banded near the top of standard Ficoll gradients, well above the banding position of the majority of cells. It was predicted that if, after infection, M cells were contributing to the profile shift, then an enrichment of M cells should be found near the top of this gradient, an enrichment of even greater magnitude than that inherently found in this region in an uninfected

log phase population (see Figure 21).

An experiment was designed to investigate the contribution of M phase cells to the shift. In this experiment two L-M cell cultures were employed. One was infected with vaccinia virus, at an MOI=1, and the other was mock-infected. At 3 hours PI aliquots from each culture were analyzed on standard Ficoll gradients. Each fraction was analyzed for both cell count and mitotic index. Results are plotted in Figure 25.

As in Figure 20, the cell distribution profiles of the mock-infected and virus-infected cultures differed somewhat at 3 hours PI, with the main cell peak of the mock-infected culture being about 2 fractions lower in the gradient than the main cell peak of the virus-infected culture. Both populations as a whole had approximately equal mitotic indexes, with a value of under 1%. In Figure 25 it can be seen that, for the mock-infected culture, fraction 5 contained a population of cells more highly enriched in M phase members than any other fraction (mitotic index = 1.8%). For the virus-infected culture, the corresponding population was found in fraction 3 (mitotic index = 4.5%).

The 2.5-fold enrichment of M cells (1.8% to 4.5%) in fraction 3 (virus-infected) over fraction 5 (mock-

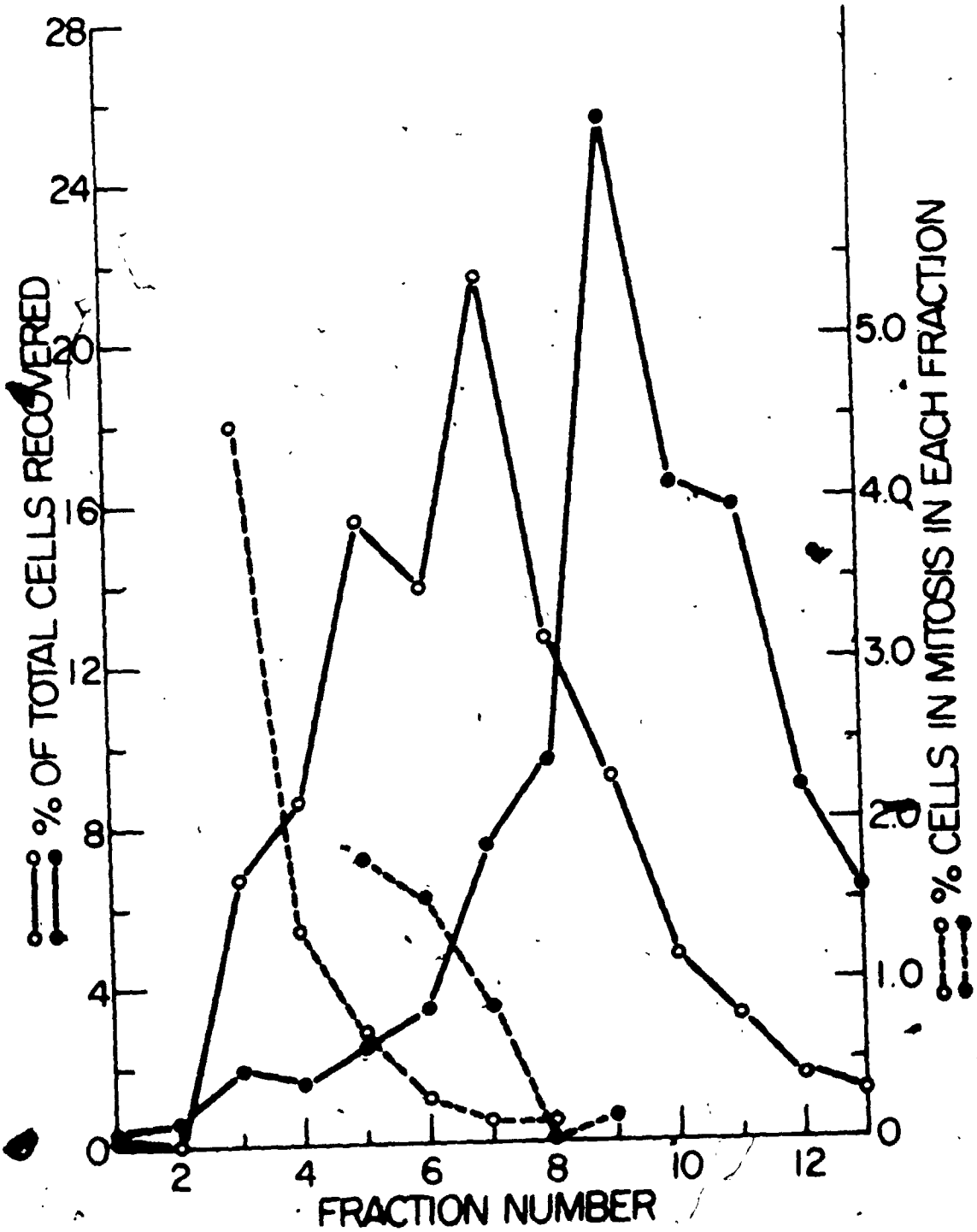
FIGURE 25

LOCATION OF M PHASE VACCINIA VIRUS-INFECTED SUSPENSION CULTURE L-M CELLS IN A FICOLL GRADIENT, AT 3 HOURS PI AT AN MOI OF 1

Conditions for infection and analysis were as described in Materials and Methods. Cell yields were: 97% (virus-infected); 45% (mock-infected).

		Total number of cells recovered
o—●—o	Cell distribution profile of virus-infected cells, and	4.0×10^5
o- - -o	the percent of these cells showing mitotic figures in each fraction.	
●——●	Cell distribution profile of mock-infected cells, and	8.5×10^5
●- - -●	the percent of these cells showing mitotic figures in each fraction.	

FIGURE 25


28
24
20
16
12
8
4
0
% OF TOTAL CELLS RECOVERED
5.0
4.0
3.0
2.0
1.0
0
% CELLS IN MITOSIS IN EACH FRACTION
2
4
6
8
10
12
FRACTION NUMBER

infected) led to the conclusion that cells in M phase *at the time of analysis* (not necessarily at the time of infection) were definitely being recruited into a population of cells which exhibited an altered sedimentation property in a Ficoll gradient, and in this way were contributing to the phenomenon. Unlike the results with S phase cells, this contribution was evident by 3 hours PI, in spite of the low MOI used.

S) LENGTHS OF THE 4 PHASES OF THE L-M CELL CYCLE

An experiment was performed to determine the approximate lengths of the M, G_1, S, and G_2 phases of the cell cycle. After pulsing a log phase suspension population with 5 μCi/ml of ^{3}H-TdR for one hour, the radioactive medium was removed and replaced with medium containing 2 μgm/ml colcemid. The culture was incubated for another 22 hours.

Between 0 and 10 hours post pulse, aliquots were removed every 2-2.5 hours for analysis of mitotic index (by fixing and staining cells, followed by phase contrast light microscopy) and mitotic cells with silver grains (by autoradiography, followed by phase contrast light microscopy). Based on the formula outlined in Appendix 4 the following results were obtained:

t_{G_1} = 7.5 hours, t_S = 10.4 hours, t_{G_2} = 8.0 hours, t_M = 0.1 hours.

DISCUSSION

The results reported in this study, when taken collectively, would seem to indicate that the alteration in the sedimentation property of L-M cells in a Ficoll gradient arising from infection with vaccinia virus can be considered as an "early" phenomenon, resulting from the synthesis of some early protein(s) which is translated from an early mRNA species transcribed after virus infection. The trivial explanation that the phenomenon is simply a result of the act of phagocytosis, whereby virus particles are taken into cells, is ruled out on the basis of the finding that when inert latex particles of very similar size to vaccinia virions are mixed with cells (and presumably phagocytized) the density gradient shift phenomenon fails to occur.

In subsequent paragraphs the reader will be presented with a summation of the results obtained in this study, an interpretation of them, and the rationale for doing the experiments which gave these results. Finally, a section will be presented which discusses the possible applications of the gradient separation technique used in this study with virus-infected cells.

C) BASIS FOR EXAMINING THE DISTRIBUTION PROFILES OF VACCINIA VIRUS-INFECTED CELLS, AND MOCK-INFECTED CELLS, EARLY AFTER INFECTION

The first report of the separation of virus-infected cells from uninfected cells on density gradients was made by Sykes *et al.* (1970), in a study basically designed to separate epithelial tumour cells from fibroblast tumour cells. Almost as an aside, these investigators attempted to apply the technique they were using - discontinuous Ficoll gradient centrifugation - to virus-infected cell cultures.

They examined the following virus-cell combinations: vaccinia virus, poliovirus I, echovirus (type 1), and influenza A_o-WSN (in its 13th passage) with ME-180 cells (metastatic carcinoma of the cervix); and vaccinia virus with CMP cells (adenocarcinoma of the bowel). Monolayer cultures were used throughout, and were trypsinized prior to sedimentation analysis. The titer of the viruses was unknown, but in each case sufficient virus was used to produce gross CPE within 7 days but not within 2 days.

Sykes and co-workers subjected virus-infected cultures to discontinuous Ficoll gradient centrifugation. In the case of vaccinia virus-, poliovirus-, and echovirus-infected cultures at 48 hours PI, cells banding in the upper zones of the gradient showed massive CPE

A) BASIS FOR CHOICE OF FICOLL AS MATERIAL FOR DENSITY GRADIENTS

Over the past nine years a number of different substances have been used in making density gradients for the separation of mammalian cells. Sinclair and Bishop (1965), who were the first to separate mammalian cells following the technique developed by Mitchison and Vincent (1965), used gradients which consisted of 5-15% plain sucrose. Later Schindler *et al.* (1970) modified the gradient by using *isotonic* sucrose, in complete medium. Other substances which have been used to make density gradients include colloidal silica (Pertoft and Laurent, 1969), fetal calf serum in phosphate buffered saline (MacDonald and Miller, 1970), gum acacia (Sugawara *et al.*, 1971), and bovine serum albumin (Leif and Vinograd, 1964). One of the first reported uses of Ficoll gradients was made by Morris *et al.* (1967). Although they found essentially the same distribution of cells and radioactivity in Ficoll gradients as in sucrose gradients, Ficoll is usually considered to be more advantageous to use in separating

mammalian cells than sucrose, for reasons outlined below.

Ficoll is a synthetic polymer of sucrose, made by copolymerizing sucrose and epichlorohydrin. Its weight average molecular weight is 400,000 ± 100,000. Unlike sucrose solutions, which for the densities required to separate mammalian cells have relatively high osmotic pressures, solutions of Ficoll with these required densities are found to be very low in both osmotic pressure and viscosity. Since mammalian cells are known to be rather susceptible to changes in the osmotic pressure of their aqueous environment, Ficoll solutions seem to have an advantage over solutions of other compounds (e.g. sucrose) in minimizing such changes.

Additional advantages of Ficoll are its inertness (no ionized groups), its inability to normally penetrate membranes (due to its colloidal nature), its ability to withstand autoclaving, its stability in neutral solutions, and its solubility in aqueous media.

B) ESTABLISHMENT OF FICOLL GRADIENT CONCENTRATION RANGE

This investigation began with an attempt to repeat the results obtained by Creighton (1970). Using the identical cells and growth medium, and following the protocol outlined by Creighton, this investigator tried

repeatedly to obtain discrete subpopulations of L-M cells banding in unique regions in a 15-22% w/v continuous Ficoll gradient. All attempts were unsuccessful, however, since most cells which were centrifuged were invariably found to band in a single zone of the gradient with a concentration of Ficoll of approximately 18.3%.

Two possible explanations which can be put forth in an attempt to explain these discrepant results are as follows: First, the manner in which the cells were manipulated prior to centrifugation might have differed unknowingly between investigators; and secondly, the L-M cells themselves might have been behaving differently in the two studies. This latter explanation is probably more reasonable than it would at first appear, in view of the careful observations made of the behaviour of the L-M cell over many years by various personnel in this laboratory (Dr. E. L. Medzon, personal communication), which indicate that the L-M cell experiences regular, fairly serious, "crises" in its growth, at times in the year perfectly coincident with changes in the season. Any one of a number of factors might be responsible for this observed phenomenon, including changes in the relative humidity in the laboratory and in the quality of the water used to make medium and wash glassware. Finally it should be mentioned that Creighton's cells were obtained from Merchant, whereas cells used in this study were purchased from the American Type Culture Collection. The possibility

therefore exists that the cells were not identical in these studies.

As oultined in Results section A), a second 60 minute centrifugation of L-M cells in a 17.2%:18.4%: 20.4% discontinuous Ficoll gradient moved the entire cell banding pattern in the gradient down. This observation suggested that the basis of the separation might not be buoyant density (and hence would probably be sedimentation-rate), since with buoyant density separation once isopycnic banding has occurred no further repositioning of cells takes place upon additional centrifugation (see Historical Review section D1). However it was possible that the observation described above was made under conditions which were insufficient to achieve isopycnic banding of cells (i.e. insufficient time and/or gravitational force). Therefore the possibility that the basis of the separation might be buoyant density after all can not be entirely discounted. (See Discussion section E)).

The objective in determining the maximum and minimum concentrations of Ficoll to use in the gradient was to maximally achieve separation of the cell population. Furthermore, it was hoped that the cell distribution profile resulting from a maximally separated population would take the approximate shape of a normal distribution "bell" curve, for previous studies relating cell size to number of cells in a given size class (for suspension cultures) had shown that the relationship

between these two parameters does indeed take the form of a bell-shaped curve (e.g. Anderson *et al.*, 1969; Schindler *et al.*, 1970). As the data in figures 2 and 3 show, the best maximum and minimum concentrations of Ficoll for a bell-shaped distribution profile were found to be 19% w/v and 14% w/v, respectively.

Using 14-19% continuous gradients it was observed that cells located throughout almost all of the gradient (see Figure 3), including regions at or very near the bottom. In switching from continuous to discontinuous gradients, however, it was found that very few, if any, cells entered the 19% w/v Ficoll layer. The only way this investigator could reconcile these facts was to assume that the L-M suspension culture cells were able to band in concentrations of Ficoll less than, but not equal to, 19% w/v. In the case of the continuous Ficoll gradient, only the Ficoll at the absolute bottom of the gradient would have this concentration, whereas in the case of the discontinuous 14-19% Ficoll gradient, 1/6 of the volume of the entire gradient contained 19% w/v Ficoll.

C) BASIS FOR EXAMINING THE DISTRIBUTION PROFILES OF VACCINIA VIRUS-INFECTED CELLS, AND MOCK-INFECTED CELLS, EARLY AFTER INFECTION

The first report of the separation of virus-infected cells from uninfected cells on density gradients was made by Sykes *et al.* (1970), in a study basically designed to separate epithelial tumour cells from fibroblast tumour cells. Almost as an aside, these investigators attempted to apply the technique they were using - discontinuous Ficoll gradient centrifugation - to virus-infected cell cultures.

They examined the following virus-cell combinations: vaccinia virus, poliovirus I, echovirus (type 1), and influenza A_o-WSN (in its 13th passage) with ME-180 cells (metastatic carcinoma of the cervix); and vaccinia virus with CMP cells (adenocarcinoma of the bowel). Monolayer cultures were used throughout, and were trypsinized prior to sedimentation analysis. The titer of the viruses was unknown, but in each case sufficient virus was used to produce gross CPE within 7 days but not within 2 days.

Sykes and co-workers subjected virus-infected cultures to discontinuous Ficoll gradient centrifugation. In the case of vaccinia virus-, poliovirus-, and echovirus-infected cultures at 48 hours PI, cells banding in the upper zones of the gradient showed massive CPE

4 days after being cultured from the gradient, whereas cells banding in the lowest zone showed no CPE for up to 10 days after being cultured from the gradient. In the case of influenza virus-infected ME-180 cultures, cells from the upper zones were found to be hemadsorption positive 8 days after being cultured, whereas cells from the lower zones were negative 21 days after culture.

At 48 hours PI, vaccinia virus-infected cells were analyzed for their sedimentation distribution. These investigators found an almost 2-fold increase in the percentage of cells in the topmost zone of their discontinuous Ficoll gradients with vaccinia virus-infected cultures, as compared to uninfected control cultures.

Vaccinia virus-induced CPE in tissue culture cells is considered to consist of 2 distinct stages (Appleyard *et al.*, 1962). The first stage, occurring prior to viral multiplication, takes the form of cell rounding, followed much later (after virus multiplication has occurred) by cell fusion. Since the cell rounding CPE has been occasionally observed to occur as early as 45 minutes PI, and in any case usually by 2 hours PI (Brown *et al.*, 1959), it seemed reasonable to test the hypothesis that those vaccinia virus-infected cells in a population which demonstrate cytopathology should be separable *early* after infection from those cells in

the population which, at the time of analysis (and for whatever the reason), lack evidence of cytopathology. The results of the sedimentation analysis at 48 hours PI obtained by Sykes *et al.* (1970) suggested the possibility - if not the likelihood - of success in attempting to substantiate this hypothesis.

D) CHARACTERIZATION OF THE VACCINIA VIRUS-INDUCED CELL SEDIMENTATION DISTRIBUTION ALTERATION

The results presented in Figures 5 and 6 allow the conclusion to be drawn that the alteration in the cell sedimentation distribution pattern, which takes the form of a shift upwards in the discontinuous Ficoll gradient, is a time-dependent and MOI-dependent phenomenon.

From Figure 5 it can be seen that the distribution profile shift can be detected as early as 1 hour PI, at an MOI of 10, and that by 2-3 hours PI most cells which are going to shift in position have already done so. (Using MOI values of 0.1 or 0.5 (results not shown) the shift is undetectable at 2-3 hours PI, but becomes evident by 8 hours PI). From Figure 6 it can be seen that the degree of participation in the shift by cells from the population increases with increasing amounts of virus added. This finding is consistent with the theory that

those cells of a population which assume the altered sedimentation characteristic are in fact the virus-infected members. Proof of this theory, however, necessitated an analysis of the infectivity of cells in different fractions from a Ficoll gradient.

The results of such an infectious center assay, seen in Figure 9, demonstrated that the "shifted" cell population contained approximately 4 times as many infected members as the "unshifted" population. Thus a definite enrichment of virus-producing cells had occurred in a fraction near the top of the gradient. Lower fractions in the gradient, containing the majority of cells, did contain a significant percentage (11%) of virus-infected cells, however. Why these infected cells had not assumed the altered sedimentation property is not clear, but one possibility is that virus replication in these infected but unshifted cells had not yet reached that critical stage at which the sedimentation change occurs. Although steps were in fact taken to synchronize the infection (virus adsorption at 4°C prior to penetration at 37°C), other factors such as cells being at different stages in their traverse around the cell cycle could account for the virus replicating asynchronously from cell to cell. This explanation is quite reasonable in view of the reports of Groyon and Kniazeff (1967) and Mantani and

Kato (1972) that, for example, cells in M phase at the time of infection do not replicate vaccinia virus or cowpox virus.

E) BIOPHYSICAL NATURE OF THE CELL CHANGE LEADING TO THE ALTERED SEDIMENTATION DISTRIBUTION

Moss *et al.* (1971) reported that within 1-2 hours after infection of HeLa suspension culture cells or chick embryo fibroblast monolayer culture cells with vaccinia virus, at an MOI of 30, virus-induced glycoproteins were synthesized and were found specifically associated with cell membranes. Very recently Weintraub and Dales (1974) obtained similar results using HeLa monolayer culture cells infected with vaccinia virus at an MOI of 10, except that they were unable to detect virus-specific glycoprotein in plasma membrane preparations prior to 8 hours PI. Fenner *et al.* (1974) have suggested that the introduction of these new glycoproteins into cell surfaces might by responsible for the morphological changes that take place after infection with vaccinia virus. Certainly the timing of the occurrence of the virus-induced glycoprotein synthesis described by Moss and co-workers agrees well with the timing of the occurrence of cell rounding CPE as described by Bablanian (1968) and others..

From the results already obtained in this study regarding the Ficoll gradient sedimentation distribution change of infected L-M cells, it was predicted that the nature of this change might be an alteration in the density and/or volume of these cells. This prediction seemed reasonable in view of the findings of Moss *et al.* as mentioned above, in that it seemed plausible that an alteration in the glycoprotein content of cell plasma membranes might result in an overall density and/or volume change of such affected cells. As already mentioned (section B) above), an observation was made early on in this study which suggested that the basis of the gradient centrifugation separation might not be buoyant density differences of cells but rather sedimentation-rate differences.

With all these points in mind, two types of analysis were carried out to examine infected and uninfected cells for size. The results, found in Figures 7 and 8, indicated that infected cells were up to 13% *larger* in volume than uninfected cells. This fact therefore discounted the likelihood of the basis of the separation being sedimentation-rate (since the larger a cell the *further* - not less - it should sediment in a gradient, according to this theory), and left buoyant density as the more likely basis. However, if buoyant density in fact was the underlying principle of the separation in Ficoll gradients described in this

thesis, then the observation discussed in section B) above, and described in detail in Results section A), must have been due to insufficient centrifugation time and/or centrifugal force to reach equilibrium conditions. The relationship between cell volume increase and cell density decrease can be found outlined in Appendix 7.

F) INHIBITION OF THE VACCINIA VIRUS-INDUCED CELL SEDIMENTATION DISTRIBUTION ALTERATION

The approach taken in this study in attempting to discover the mechanisms leading to the sedimentation distribution shift was to try to inhibit the occurrence of the phenomenon either by inactivating the virus prior to infecting cells or by adding inhibitors of macromolecular synthesis to cells infected with active virus. By knowing the modes of action of these inactivating agents and inhibitors, one can infer from the results obtained what the likely mechanisms might be which give rise to the phenomenon.

The agents chosen in this study to inactivate virus were heat and ultra-violet irradiation; virus-specific antibody was used to neutralize virus. Cytosine arabinoside was chosen to inhibit DNA dependent DNA synthesis, actinomycin D to inhibit DNA dependent RNA synthesis, and cycloheximide to inhibit protein synthesis.

(i) Effect of cytosine arabinoside

The results depicted in Figure 10 show clearly that 10 µgm/ml ara C, present from 0 hour PI through to 2.5 hours PI, was not able to inhibit the occurrence of the vaccinia virus-induced sedimentation profile shift of L-M cells in a Ficoll gradient, in spite of being able to inhibit DNA synthesis (as measured by incorporation of ^{3}H-TdR into TCA precipitable material) by more than 95%.

The conclusion drawn from the results in Figure 10 was that the virus-induced cell sedimentation distribution shift of L-M cells was not dependent on DNA dependent DNA synthesis.

(ii) Effect of actinomycin D

The results depicted in Figure 11 suggest that 10 µgm/ml actinomycin D, present from 0 hour PI through to 2.5 hours PI, was able to at least partially inhibit the occurrence of the vaccinia virus-induced sedimentation shift of L-M cells, in spite of only being able to inhibit RNA synthesis (as measured by incorporation of ^{3}H-UR into TCA precipitable material) by approximately 83%.

This partial inhibition of the shift took one

of two forms: either an intermediate *position* of cells in a Ficoll gradient, between the "shifted" and "unshifted" positions, when 3 µgm/ml of inhibitor was used (results not shown); or, as exemplified in Figure 11, an intermediate *contribution* of cells in a Ficoll gradient to the "shifted" and "unshifted" populations, when 10 µgm/ml of inhibitor was used.

Dales (1964) examined by electron microscopy the fate of vaccinia virions in L cells treated with 1 µgm/ml and 3 µgm/ml actinomycin D. With the lower concentration of inhibitor he reported that virus penetration, primary uncoating, and secondary uncoating proceeded normally; with the higher concentration of drug virus penetration and primary uncoating proceeded normally (i.e. *early* genome functioning was not inhibited), but secondary uncoating was reduced by 80-90%. Presumably 10 µgm/ml actinomycin D would not result in a decrease in the inhibition of secondary uncoating (if anything an increase in inhibition would be expected), and furthermore hopefully would inhibit primary uncoating as well. If in fact this were the case then one could conclude that 10 µgm/ml actinomycin D would be inhibiting *early* genome functioning as well as late genome functioning, providing that virus penetration was proceedingly normally. The data obtained

by Harry (personal communication) suggests that adsorption to and penetration of L-M cells by vaccinia virus is not inhibited by 10 µgm/ml actinomycin D. Upon treating virus-infected cells in the presence of the drug with ^{125}I-labelled anti-vaccinia virus IgG, he finds a significant drop by 15 minutes PI in the amount of antibody bound to the surface of the cells, suggesting that input virions have successfully penetrated the cells.

Using a different approach, Metz and Esteban (1972) showed that a low dose of actinomycin D (1 µgm/ml) was ineffective in inhibiting early cytoplasmic RNA synthesis (viral specific mRNA?) in vaccinia virus-infected L cells, presumably because the drug could not penetrate the core structure. Again one would hope that a ten-fold increase in the concentration of this drug would lead to inhibition of synthesis of this species of RNA, much as cordycepin does (Esteban and Metz, 1973).

The conclusion drawn from these data was that the virus-induced cell sedimentation distribution shift of L-M cells was dependent to some extent on DNA dependent RNA synthesis, which might occur at the primary stage of uncoating.

(iii) Effect of cycloheximide

The results depicted in Figure 12 show clearly

that 10 µgm/ml cycloheximide, present from 0 hour PI through to 2.5 hours PI, inhibited the occurrence of the vaccinia virus-induced sedimentation profile shift of L-M cells, under conditions in which protein synthesis (as measured by incorporation of ^{3}H-amino acid mixture into TCA precipitable material) was inhibited by 95%. The trivial explanation that this result was due to the inability of the virus to adsorb to and penetrate the cell in the presence of cycloheximide was ruled out on the basis of the data of Harry (personal communication), who obtained results with 10 µgm/ml cycloheximide much like those he obtained with actinomycin D (see (ii) above).

The conclusion drawn from the results in Figure 12 was that the virus-induced cell sedimentation distribution shift of L-M cells was dependent on protein synthesis.

(iv) Effect of ultra-violet inactivation of vaccinia virus

The results depicted in Figure 14 clearly show that UV-inactivated vaccinia virus was not able to induce the characteristic sedimentation shift of L-M cells in a Ficoll gradient. The titer of the virus was found to have

dropped by 99% as a result of UV inactivation.

As mentioned in Results section J), the fate of UV-irradiated vaccinia virus in infected cells is by no means clear. Harry has evidence (personal communication) which suggests that, under inactivation conditions similar to those used in this study, irradiated vaccinia virus adsorbs to, but does not penetrate, L-M monolayer culture cells. On the other hand Joklik (1964) presented evidence indicating that a 2.5 minute exposure of vaccinia virus to UV irradiation resulted in only a 9% decrease in primary uncoating, although secondary uncoating dropped by 97%.

It must be borne in mind in interpreting Joklik's data that he was not assaying for the primary and secondary uncoating events themselves, but rather for the *products* formed by these events (cores and DNAase-susceptible DNA, respectively). Therefore, for example, the finding that secondary uncoating dropped in cells infected with UV-inactivated vaccinia virus by 97% does not allow one to pinpoint which event(s) normally occurring between the core stage and the "viral DNA susceptible" stage is being interfered with in the case of UV-inactivated virus. Such an event might be the synthesis of early mRNA by the virion-bound DNA dependent RNA polymerase enzyme, or synthesis of the putative uncoating protein, or perhaps

the uncoating event itself, carried out by the "uncoating protein".

The conclusion which was drawn from the results in Figure 14 was that, assuming UV-inactivated virus successfully penetrated L-M cells, some event(s) occurring within the infected cell between the core stage and the "viral DNA susceptible" stage was necessary in order to induce the characteristic cell sedimentation distribution profile shift. Since the precise mode of action of UV inactivation of vaccinia virus is not known, it could not be concluded what event(s) that might be.

(v) Effect of heat inactivation of vaccinia virus

The results depicted in Figure 15 suggested that heat-inactivated vaccinia virus was unable to induce the characteristic sedimentation shift of L-M cells in a Ficoll gradient. The titer of the virus was found to have dropped by over 99% as a result of heat inactivation.

Joklik (1964) found that heating vaccinia virus at 60°C for 15 minutes resulted in primary uncoating proceeding normally but secondary uncoating decreasing by 93%. Dales and Kajioka (1964), by electron microscopy, determined that uptake of virus into

phagocytic vacuoles was not prevented, but that the normal transfer of viral cores from these vacuoles (i.e. secondary uncoating) was blocked. Under identical conditions of heat inactivation to that used in the present study, Harry (personal communication) obtained evidence which indicates that heat-inactivated vaccinia virus penetrates L-M monolayer cells, but only slightly as compared to unheated control virus.

In any case, the conclusion drawn from the results in Figure 15 was that, assuming that heat-inactivated virus successfully penetrated L-M cells, some heat-labile constituent of the virion was necessary in order to induce the characteristic cell sedimentation distribution profile shift. A likely candidate for this putative heat-labile constituent is the virion-bound DNA dependent RNA polymerase enzyme, as mentioned in Results, section K).

(vi) Effect of antibody neutralization of vaccinia virus

The results depicted in Figure 16 clearly showed that antibody-neutralized vaccinia virus was able to induce the characteristic sedimentation shift of L-M cells in a Ficoll gradient. The titer of the virus

was found to have dropped by 95-99% as a result of antibody neutralization.

Joklik (1964) found that antibody neutralization of vaccinia virus to the extent of 92-97% resulted in only 31% of the adsorbed virions (vs. 84% with the unneutralized control virus) successfully penetrating HeLa cells. Those virions which were successfully phagocytized were uncoated to the core stage, but did not undergo secondary uncoating. Similar results were obtained by Dales and Kajioka (1964) except that they found that "the majority" of adsorbed virions successfully penetrated L cells. The discrepancy between these two results might have been due to the different cell lines used. Of some interest is the recent electron microscopical data obtained by Greer *et al.* (1974), which demonstrated that immune rabbit macrophages were able to phagocytize vaccinia virus equally as well as nonimmune macrophages, and that in addition the immune macrophages were able to process the virus through the first stage of uncoating. The failure of vaccinia virus to replicate in immune macrophages was attributed by Greer *et al.* to a failure within the immune macrophages of some mechanism operating at a point beyond first stage uncoating. Since no examination was performed to determine the degree of secondary uncoating, it could not be determined from this

particular study what event beyond primary uncoating failed to occur in the infected immune macrophages. The results of Joklik, Dales, and Kajioka, however, suggest that secondary uncoating did not occur in cells infected with antibody-neutralized virus.

In general it appears that the antibody-neutralized vaccinia virion is phagocytized by all types of cells (immune and none-immune macrophages, as well as cells other than macrophages), and that the protection afforded by anti-vaccinia virus antibody lies in its ability to interefere with some virus replication event occurring after primary uncoating and before secondary uncoating is complete.

The conclusion drawn from the results in Figure 16 was that the event(s) taking place within infected L-M cells which leads to the sedimentation distribution shift of these cells in a Ficoll gradient occurs after cores appear within infected cells, but prior to the parental DNA becoming susceptible to DNAase. Such an event might be the synthesis of early mRNA by the virion-bound RNA polymerase enzyme, or synthesis of the "uncoating protein". Presumably it could not be the uncoating event itself, carried out by the "uncoating protein", since this step would lead directly to the

production of DNAase susceptible viral DNA, which it has been suggested does not occur in the antibody-neutralized system.

G) OVERALL CONCLUSIONS FROM INHIBITION STUDIES

Considering all the results discussed in F) above, an overall conclusion was reached concerning the possible molecular events leading to the distribution shift. The conclusion reached was that the alteration in the sedimentation property of infected L-M cells was due to the synthesis of some protein molecule(s) translated from early mRNA, which had been transcribed from parental vaccinia virus DNA by the virion-bound RNA polymerase while the virus was at the core stage of replication. Thus the protein(s) giving rise to the sedimentation shift could be defined as an "early" protein.

The substantiation for this conclusion would be as follows:

a) Protein synthesis has been shown to be required.
b) RNA synthesis has been shown to be required.
c) DNA synthesis has been shown to be not required.
d) Heat inactivation of virus prevents the occurrence of the shift - possibly by destroying the virion-bound RNA polymerase enzyme.

e) Ultraviolet inactivation of virus prevents the occurrence of the shift-possibly by preventing synthesis of early mRNA or early protein, or the functioning of an early protein such as the "uncoating protein".

f) Antibody neutralization of virus does not prevent the occurrence of the shift-possibly because such virus is only prevented from replicating at some point in the replication cycle *after* the synthesis of the early mRNA and early protein required for the shift (e.g. the uncoating event itself).

Points a) through f) can be found summarized schematically in Tables 2 and 3.

H) SUPPORT FOR THE CONCLUSION REACHED IN SECTION G) REGARDING THE PROPOSED MECHANISM OF THE ALTERATION IN THE SEDIMENTATION PROPERTY OF VACCINIA VIRUS-INFECTED L-M CELLS IN A FICOLL GRADIENT

After carefully searching through the literature, a comparison of published data has revealed to this investigator that good agreement exists between the early change induced in L-M cells (as described herein) and a number of other early vaccinia virus-induced changes (as described in the literature), in terms of the dependencies of these changes on macromolecular syntheses and the effects of inactivated virus on these changes.

For example, Bablanian (1968), in studying

early vaccinia virus-induced cell rounding CPE in LLC-MK2 cells, using an MOI of 50, found the following:

(i) The presence of 2.5 µgm/ml 5-fluoro-2'-deoxyuridine (FUdR) (which inhibits DNA synthesis) did not affect the induction of normal amounts of CPE (100% of cells damaged by 3 hours PI).

(ii) The presence of 5 µgm/ml actinomycin D reduced cell rounding CPE by more than 90%.

(iii) The presence of 330 µgm/ml puromycin (which inhibits protein synthesis) completely prevented cell rounding CPE.

(iv) UV-irradiated virus induced much less cell rounding CPE than unirradiated control virus.

(v) Heat-inactivated virus was unable to induce cell rounding CPE. Bablanian's conclusion was that the early vaccinia virus-induced cell rounding CPE was due to the active synthesis of one or several virus-induced proteins.

Ueda *et al.* (1969), in studying the induction of early vaccinia virus-induced antigens on the surface of infected HeLa cells by immunofluorescence, using an approximate MOI of 1, found the following:

(i) The presence of 20 µgm/ml ara C did not affect the induction of normal amounts of antigen on the cell surface.

(ii) The presence of 20 µgm/ml cycloheximide completely prevented the induction of antigen on the cell surface.

Ueda and co-workers' conclusion was that the early vaccinia virus-induced surface antigen synthesized in infected cells was a newly synthesized protein coded by an early function of the viral genome.

Miyamoto and Kato (1971), in studying the induction of early cowpox-induced antigens on the surface of infected FL (human amnion) cells by immune hemadsorption and fluorescent antibody technique without fixation, using an MOI of 1-10, found the following:

(i) Formation of the cell surface antigen was not inhibited by the presence of 10 µgm/ml ara C or 10^{-5} M FUdR.

(ii) No cell surface antigen was detected when 5 µgm/ml actinomycin S was added any time prior to 1 hour PI.

(iii) No cell surface antigen was detected when 20 µgm/ml puromycin was added any time prior to 3 hours PI.

The conclusion drawn by Miyamoto and Kato was that the virus-induced cell surface antigen they were studying might have been a newly synthesized protein which was translated during the third hour after viral infection, and that its synthesis was mediated by newly synthesized

RNA transcribed from the viral DNA during the first hour PI.

Zarling and Tevethia (1971), in studying the early vaccinia virus-induced expression of Concanavalin A (Con. A) binding sites on rabbit kidney cell surfaces as measured by agglutination, using an MOI of 3, found the following:

(i) Heat-inactivated virus was unable to induce the expression of Con. A binding sites.

(ii) Antibody-neutralized virus was unable to induce the expression of Con. A binding sites.

(iii) The presence of 20 µgm/ml ara C from 6 hours pre-infection did not inhibit the expression of Con. A receptors.

(iv) The presence of 25 µgm/ml cycloheximide, added at any time up to 2 hours PI, prevented the expression of Con. A receptors.

The conclusion which Zarling and Tevethia drew was that DNA synthesis was not a prerequisite for this cell surface alteration, but that *de novo* protein synthesis was required for the expression of Con. A binding sites, and that it occurred between 0 and 2 hours PI.

Harry (personal communication), in

studying the early vaccinia virus-induced appearance of virus-specific antigen(s) on the surface of L-M monolayer cells by radioimmunoassay, using an MOI of 1, finds the following:

(i) The presence of 10 µgm/ml ara C only inhibited partially the appearance of the antigen.

(ii) The presence of 10 µgm/ml actinomycin D between 30 minutes and 90 minutes PI inhibited the appearance of this antigen.

(iii) The presence of 10 µgm/ml cycloheximide inhibited the appearance of this antigen.

(iv) Heat-inactivated virus was incapable of inducing the antigen.

Harry's conclusion is that the appearance of the virus-specific antigen he was studying is only partly dependent on DNA synthesis (i.e. partly early coded, and partly late coded), is dependent on RNA synthesis, which must be occurring between 30 and 90 minutes PI, and is dependent on protein synthesis. Finally it appears as though a functioning virion-bound RNA polymerase is required in order that the antigen be synthesized.

Finally, Faught (1974) studied the early vaccinia virus-induced activation of an acetylesterase in infected L-M suspension culture cells. He found the

phenomenon to have the following characteristics:

(i) The presence of 3 µgm/ml actinomycin D did not inhibit the activation of this enzyme.

(ii) The presence of 10 µgm/ml cycloheximide prevented the activation of the enzyme.

(iii) Heat-inactivated virus was able to induce the activation of the enzyme.

(iv) UV-inactivated virus was able to induce the activation of the enzyme.

The conclusion which Faught drew was that activation of acetylesterase in vaccinia virus-infected L-M cells was due to the action of a protein synthesized within the cell after infection. Furthermore, this protein was concluded to have been synthesized from a stable pre-existing host cell mRNA molecule, since:

(a) *de novo* RNA synthesis was not required (therefore the protein must be translated from a stable, pre-existing mRNA species); and

(b) heat and UV inactivation could not inhibit the activation (therefore the protein or mRNA involved in the activation could not be viral encoded).

The characteristics of this phenomenon seem to suggest its unrelatedness to the others listed above.

All the supportive data outlined in this section, along with the data obtained in the present study, can be found summarized in Table 1. The similarities between these various virus-induced phenomena are readily apparent. For example, all phenomena have been shown to be independent of DNA synthesis (some of the later antigenic expression detected by Harry required DNA synthesis, however), dependent on RNA synthesis (those examined), and dependent on protein synthesis. UV-inactivated virus has been found to cause markedly reduced amounts of early cell rounding CPE and to be unable to induce the early change in the sedimentation property of cells in a Ficoll gradient. Heat-inactivated virus has been found to be unable to induce all those changes which have been examined.

The only possible discrepancy in the results lies in the fact that the expression of Concanavalin A binding sites was found to be inhibited by antibody-neutralized virus, whereas the sedimentation change still occurred. Since no information was given concerning the titer of the rabbit hyperimmune serum used in the Con.A study, or whether adsorption and penetration were successful in the presence of the serum, it is possible that Con. A binding sites did not appear with antibody-neutralized virus because of an interference with phagocytosis.

TABLE 1

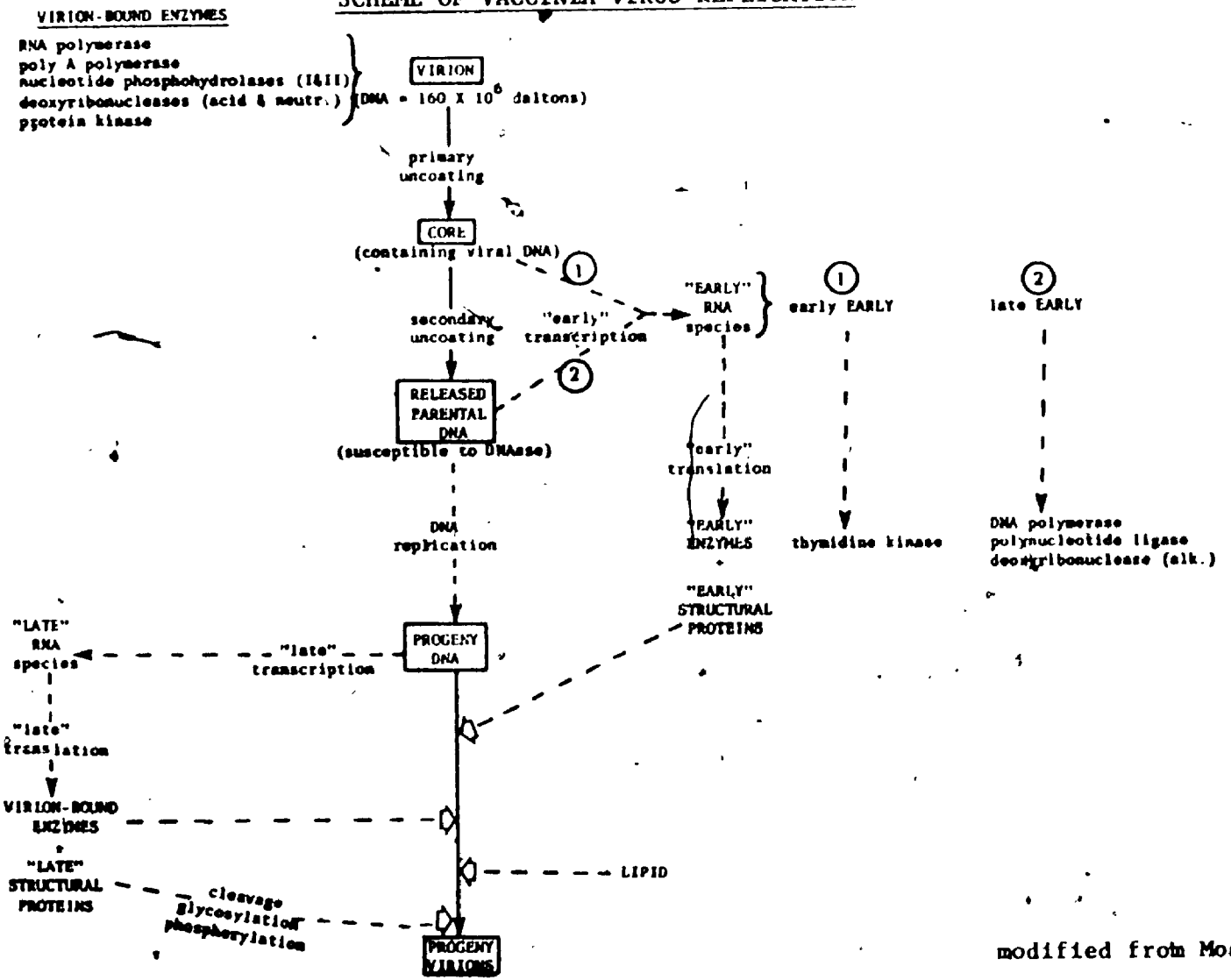
KEY: + = phenomenon occurs
- = phenomenon fails to occur
N.D. = not done

OCCURRENCE OF EARLY VACCINIA VIRUS-INDUCED CHANGES IN INFECTED TISSUE CULTURE CELLS

VIRUS-INDUCED PHENOMENON	ARA C or FUdR	ACTINOMYCIN D or S	CYCLOHEXIMIDE or PUROMYCIN	HEAT-INACT. VIRUS	UV-INACT. VIRUS	ANTIBODY-NEUTR. VIRUS
cell rounding (Bablanian, (1968)	+	+by>90%	-	-	+by>60%	N.D.
viral antigens on cell surface, by immunofluorescence (Ueda et al., (1969)	+	N.D.	-	N.D.	N.D.	N.D.
viral antigens on cell surface, by immune hemadsorption and fluorescent antibody (Miyamoto and Kato, 1971)	+	-	-	N.D.	N.D.	N.D.
Con.A binding sites on cell surface (Zarling and Tevethia, 1971)	+	N.D.	-	-	N.D.	-
viral antigens on cell surface, by radioimmunoassay (Harry, personal communication)	Partial	-	-	-	N.D.	N.D.
sedimentation change of infected cells (Hall, this study)	+	-	-	-	-	+

TABLE 2

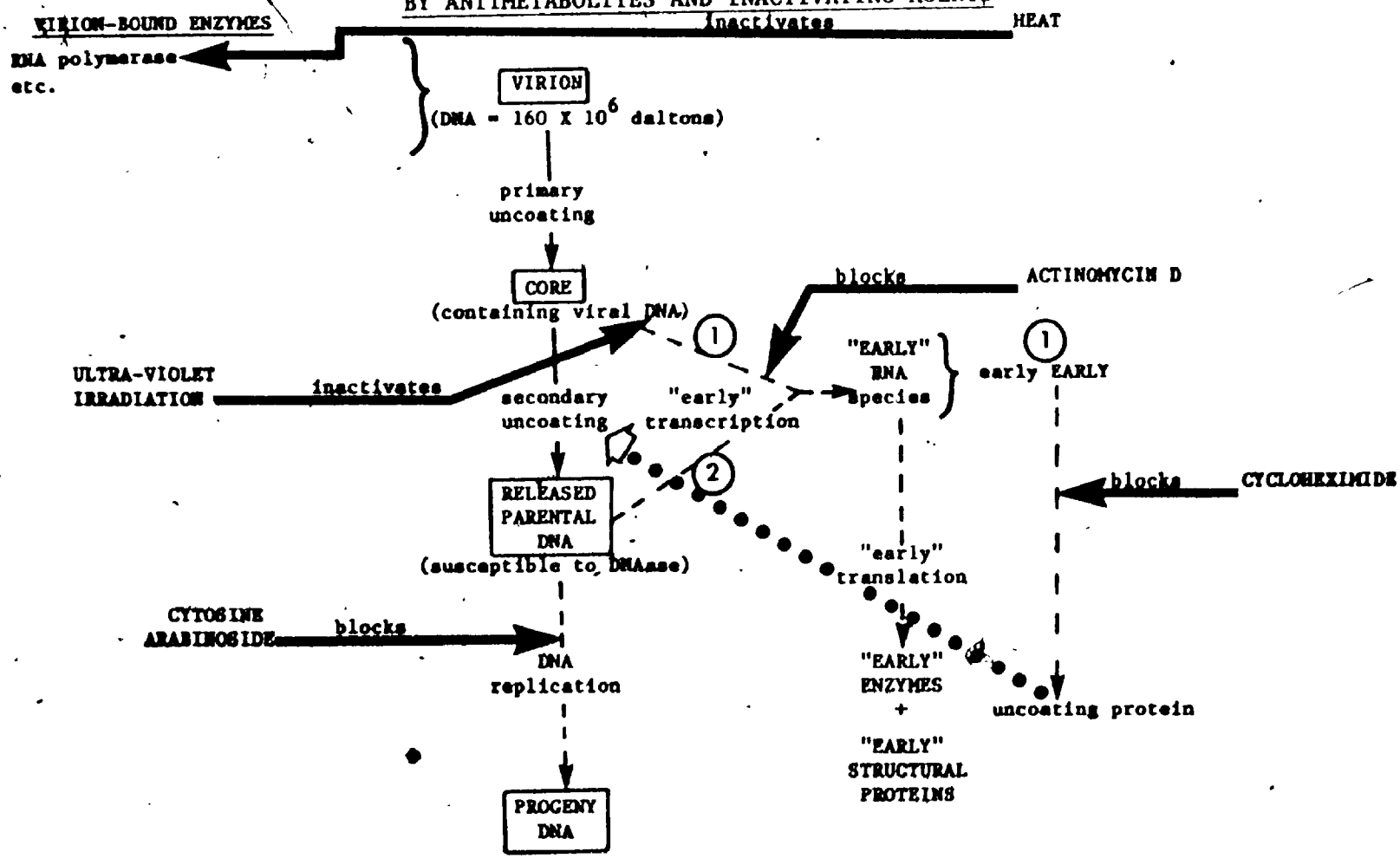
SCHEME OF VACCINIA VIRUS REPLICATION



modified from Moss (1974)

TABLE 3

EARLY EVENTS IN VACCINIA VIRUS REPLICATION & THEIR INHIBITION BY ANTIMETABOLITES AND INACTIVATING AGENTS



I) INFORMATION FOR THE SEDIMENTATION ALTERATION PROTEIN(S)- VIRUS OR CELL ENCODED?

The data obtained in this study does not allow for a differentiation between the possibilities that the RNA and protein species required to obtain the altered sedimentation property are encoded within the host cell genome or the viral genome.

One of the best ways that such a differentiation could have been made would have been to use a vaccinia virus mutant with a defective virion-bound RNA polymerase enzyme. Through the kind co-operation of Dr. W.K. Joklik, this investigator was able to obtain a vaccinia virus early function temperature-sensitive mutant, designated ts20.165 (Basilico and Joklik, 1968), which Basilico and Joklik have shown has associated with it only about 40% of the normal amount of the RNA polymerizing activity. Apparently uncoating of this mutant occurs normally but decreased amounts of early mRNA are formed (about 25% as much as formed with wild type virus), and one of the proteins formed from this early mRNA - DNA polymerase - is about twice as heat labile as the wild type polymerase.

In spite of a considerable expenditure of time and energy, this investigator abandoned work with ts20.165

due to insurmountable technical problems involved in passing and titrating the mutant at the permissive temperature (31°C). This seemed to be due to the fact that L-M monolayer culture cells did not survive well at 31°C, even when grown to confluency first at 37°C.

Another way in which a differentiation could have been made between the possibilities of the sedimentation alteration protein(s) being host cell genome encoded or viral genome encoded would have been to equate the sedimentation distribution change to any one of the changes listed in Table 1 *in which viral specific antigen synthesis* is measured, for the appearance of any viral-specific antigen is almost certainly due to virus-encoded information. An attempt was indeed made to equate the sedimentation shift to Harry's cell surface vaccinia virus-specific antigen (see section H) and Table 1).

L-M suspension culture cells were infected with vaccinia virus, at an MOI of 1, at approximately 2.5 hours PI were treated with ^{125}I-labelled anti-vaccinia virus IgG, and then (after thorough washing to remove unbound IgG) were subjected to discontinuous Ficoll gradient centrifugation. The technical limitations inherent in the technique, in terms of removing all traces of unbound IgG, made interpretation of the data impossible, however.

A third way in which a differentiation can theoretically be made between a viral encoded protein and a host cell encoded protein is to selectively inhibit the synthesis of one of them. It must be borne in mind that because inhibitors such as actinomycin D and cycloheximide are *not* selective in their actions, the fact that they inhibit the virus-induced sedimentation shift reveals nothing as to the genetic source of the information for this shift. On the other hand, a selective inhibitor, such as interferon for example, can selectively prevent virus coded proteins from being produced in infected cells. Metz and Esteban (1972) found that in L cells pretreated with interferon and then infected with vaccinia virus, protein synthesis ceased as early as 20 minutes PI (although viral RNA synthesis was not inhibited). The newly synthesized polypeptides were detected by labelling with high specific activity ^{35}S-methionine, followed by polyacrylamide gel electrophoresis; they were shown to be virus coded (Esteban and Metz, 1973) by virtue of their sensitivity to cordycepin and their insensitivity to low doses of actinomycin D. (According to these authors cordycepin inhibits virus RNA synthesis and low doses of actinomycin D do not).

In the present study attempts were made to use L cell interferon, kindly supplied independently by

Drs. L. Katz and A. Boudreault, but they were abandoned in view of the exceedingly large amounts of interferon found to be required in order to obtain significant protection of L-M cells from vaccinia virus, by the criterion of plaque reduction.

Other selective inhibitors of vaccinia virus replication, such as isatin-β-thiosemicarbazone (IBT) and rifampicin, unfortunately act too late in the virus replication cycle to be of any use in a study on early induced phenomena. For example, Fenner *et al.* (1974) pointed out that vaccinia viral mRNA, early proteins, and DNA are all synthesized in the presence of IBT, and that both "early" and "late" viral mRNA's and proteins are synthesized satisfactorily in the presence of rifampicin.

The best evidence to support the idea that the protein(s) required for the occurrence of the density gradient change is a virus-specific protein, and not a cell-specific one, comes from the work of Moss (1968). He obtained data which suggested that HeLa cell protein synthesis is rapidly suppressed after infection with vaccinia virus. If the same holds in the L-M cell system, this would imply the unlikelihood of significant amounts of any proteins made in the cell soon after infection being L-M cell-coded proteins.

The results recently obtained by Stephen *et al.* (1974) support Moss' interpretation that some specific component(s) of the vaccinia virion is responsible for the cytotoxic effect observed in infected cells. This idea is contrary to the evidence obtained by Bablanian (1968), which implied that *de novo* protein synthesis was required for early CPE.

Other support for the idea that the protein(s) required in this study is a virus-coded one comes from the work of Esteban and Metz (1973). Using the technique of labelling vaccinia virus-infected cells with high specific activity ^{35}S-methionine, as mentioned in an earlier paragraph, followed by polyacrylamide gel electrophoresis, they were able to demonstrate that virus-specific proteins were synthesized in L cells as early as 20 minutes PI. This timing was much earlier than had ever been shown before. Therefore it is indeed possible that the 'sedimentation alteration' protein is a virus-coded protein, and that its synthesis begins shortly after infection, at a time (shown by Esteban and Metz) during which there occurs a burst of cytoplasmic RNA synthesis, presumably by the virion-bound RNA polymerase.

J) NATURE OF THE CELLS CONTRIBUTING TO THE "SHIFTED" POPULATION IN A FICOLL GRADIENT AFTER INFECTION

The question was asked whether *any* cell from the general infected population was able to display the altered Ficoll gradient sedimentation property, or whether specific cells from the infected population were being recruited into forming a special sub-population with this newly acquired characteristic. Data obtained early in this study (see Figure 5) suggested that, even when MOI values were adjusted such that virtually every cell in the population would receive at least one virion (MOI = 10), there were always a small but significant number of cells which did not relocate in the gradient, as mentioned in Results section O).

In view of the possibility that the cell cycle might be playing some role in vaccinia virus replication and in the separation of cells in a Ficoll gradient, as mentioned in Results section N), it was decided to study two particular classes of cells which, together with two other classes, make up the log phase population. The cells which were chosen for study were those in M phase and those in S phase. The basis for this choice was the fact that cells in these two phases of the cell cycle can be easily detected: M phase cells (by phase contrast

light microscopy) by virtue of the fact that they lack a nuclear membrane and possess mitotic figures (distinct chromosomes); S phase cells (by autoradiography or by measurement of incorporation of precursors of DNA into TCA precipitable material) by virtue of the fact that DNA is synthesized during this phase of the cycle.

The data of Figures 20 and 25, when considered together, suggest the following:

(a) Both cells in S phase at the time of infection and cells in M phase at 3 hours PI do contribute to the "shifted" population of cells in a Ficoll gradient after vaccinia virus infection.

(b) At an MOI of 1, the contribution of cells in S phase at the time of infection can be detected by 8 hours PI but not by 3 hours PI; whereas the contribution of cells in M phase at the time of analysis can be detected by 3 hours PI.

Since vaccinia virus infection evidently does not arrest cells in mitosis (mitotic index does not rise in an infected population; see Results section R)), it could be concluded that cells scored as being in M phase at 3 hours PI were in a "3 hour pre-M" phase at the time of infection. According to the lengths of the 4 phases of

the L-M cell cycle, as determined in Results section S), a point 3 hours prior to mitosis would put these cells somewhere in G2.

This data suggests the possiblity that cells in G2 at the time of infection can more rapidly contribute to the shift in the Ficoll gradient than cells in S at the time of infection. This seems quite plausible in view of the commitment of S phase cells to replicate cellular DNA. The results obtained by Eremenko *et al.* (1972) supports this concept. They found that the initial rate of poliovirus RNA synthesis (0-2 hours PI) in HeLa cells was greatest if growth was initiated near the end of the G2 phase, and slowest if growth was initiated during S phase. It might be that S phase cells allow for adsorption and penetration of vaccinia virions, but that primary uncoating is delayed for some hours (thus delaying the Ficoll gradient distribution shift), until the obligation to synthesize cellular DNA has been completed.

K) APPLICATIONS OF THE FICOLL GRADIENT CENTRIFUGATION TECHNIQUE, FOR SEPARATING VIRUS-INFECTED FROM UNINFECTED CELLS

Sykes *et al.* (1970) were among the first to demonstrate the usefulness of Ficoll gradients in

separating fibroblasts from epithelial cells originally derived from solid human tumours. They extended their results to virus-infected cells and in this regard were the first to show that Ficoll gradients also showed a potential usefulness in separating virus-infected cells from uninfected cells.

Fox and Levine (1971) employed Ficoll gradients to partially separate tumour virus SV40-infected cells (in S phase) from noninduced cells (in G1 phase). Since only some cells in a given population are induced (to synthesize DNA and divide) by this virus, Ficoll gradient separation is most useful in enriching for these induced cells.

Several months after Fox and Levine's work was published, Sugawara *et al.* (1971) reported the ability of gum acacia gradients to separate Burkitt lymphoma cells harbouring Epstein-Barr (EB) virus from uninfected cells. Since only 5-20% of the total cells contained EB virus, gum acacia separation is most useful in enriching for these infected cells.

Ball and Medzon (1973) were the first to study the ability of Ficoll gradients to separate virus-infected from uninfected cells *under conditions in which the multiplicity of infection was intentionally kept low*. They showed that Ficoll gradients could produce a

fraction of cells which was enriched with vaccinia virus-infected members by a factor of 4, and that such a phenomenon could occur as quickly as 2 hours PI.

Recently Ross and Ash (1974) reported the density decrease of Herpes simplex virus (HSV)-infected L cells as detected in a Ficoll gradient. Using an MOI of 1 they obtained virtually a complete separation of the infected population from the control population, but fell short of analyzing cells from each fraction of the gradient for their infectivity (infectious center assay). Thus no information was obtained concerning the enrichment of HSV-infected cells in specific gradient fractions.

The approach which has always been taken in our laboratory has been to perform our research as though we were working with clinical material, so that our results would be able to be extrapolated - without losing too much of their validity - to a real-life *in situ* situation of a virus infection. Consequently our approach in this study was to adjust multiplicities of infection downwards. Notice, for example, that MOI values used extensively in the present study were in the range of 0.1 - 10. We believe that "bombarding" a tissue culture cell with hundreds of plaque-forming units of

virus is *not* the way to mimic the events which occur in a viral infection *in situ*.

In spite of this self-imposed "restriction", the present study has shown that Ficoll gradient centrifugation is a powerful tool which can be used to enrich for the virus-infected members of a cell culture. Regardless of whether the sedimentation change induced in infected L-M cells by vaccinia virus represents virus coded information or cell coded information, the change itself is worthy of investigation, for pursuing such an investigation is bound to reveal much about how a virus particle - the most simple of all biological entities - can exert such tremendous effects on a mammalian cell - a most complicated and sophisticated organism indeed. It is this investigator's hope that the studies described in this thesis will act as the basis for continued investigation in this interesting area of early virus-cell interactions.

APPENDIX 1

Assuming that an L-M suspension culture cell is a sphere in shape, its volume can be expressed as $\frac{4}{3} \pi (\frac{d}{2})^3$, where d is defined as the cell diameter and π is defined as a constant, = 3.14. In this way the volume of mulberry pollen (diameter = 12.5 μ) can be calculated to be 1022 μ^3, and the volume of ragweed pollen (diameter = 19.5 μ) can be calculated to be 3880 μ^3.

Mean volumes of mock-infected and virus-infected cells were calculated in the following way: At the mid-point on the y axis of Figure 7 a horizontal line was drawn across the graph, intersecting each curve at 2 points. From the mid-point between the 2 points of intersection of the horizontal line with the mock-infected cell curve, a perpendicular was dropped to the x axis. Similarly from the mid-point between the 2 points of intersection of the horizontal line with the virus-infected cell curve, a perpendicular was dropped to the x axis.

The distance from point A to the point where each of these perpendiculars intersects the x axis was measured. These measured distances were then converted into volume values. In this way the volume of the mock-infected cells at the mid-point was found to be 2745 μ^3 and the volume of the virus-infected cells at the mid-point was found to be 3090 μ^3. Thus the increase in volume of cells as a result of virus infection was $\frac{3090 - 2745}{2745} \times 100 = 12.6\%$.

APPENDIX 2A

(see figure 18)

Fraction number	* average cpm incorporated	* average cell count X 10^5 /ml	** average cpm/cell (relative units)
1	54	0.423	12.8
2	51	0.320	15.8
3	52	0.243	21.4
4	48	0.255	18.7
5	54	0.268	20.1
6	208	4.070	5.1
7	634	1.092	58.1
8	553	0.905	61.1
9	539	1.028	52.5
10	753	1.168	64.5
11	1079	1.509	71.5
12	1651	1.816	90.9
13	2447	2.372	103.2
14	3009	2.423	124.2
15	3146	2.061	152.6
16	2637	1.692	155.9
17	1802	1.282	140.6
18	1438	1.087	132.3
19	947	0.819	115.6
20	683	0.667	102.4
21	495	0.503	98.5
22	346	0.396	87.4
23	304	0.629	48.4
24	229	0.387	59.1
25	205	0.361	56.9
26	186	0.340	54.8
27	178	0.365	48.7
28	172	0.418	41.2
29	224	0.342	65.6
30	298	0.462	64.6
31	262	0.590	44.4
32	156	0.326	47.9
33	70	0.187	37.4

* experimentally obtained data

** derived data

APPENDIX 2B

LINEAR CORRELATION COEFFICIENT COMPUTATION

In this example an attempt is being made to determine whether a good linear correlation exists between the parameters "cpm/cell" (scintillation plus Coulter counting) and "percent cells with silver grains" (autoradiography).

Frac. No.	cpm/cell (x)	x^2	% cells $\bar{c}$ silver grains (y)	y^2	xy
8	1281	1640961	12.4	153.76	15884.4
9	1140	1299600	13.3	176.89	15162
10	930	864900	2.8	7.84	2604
11	778	605284	4.1	16.81	3189.8
12	937	877969	6.6	43.56	6184.2
13	1022	1044484	2.9	8.41	2963.8
	Σ=6088	Σ=6333198	Σ=42.1	Σ=407.27	Σ=45988.2

The linear correlation coefficient, r, is computed based on the following formula:

$$r = \frac{N\Sigma xy - \Sigma x \Sigma y}{\sqrt{N\Sigma x^2 - [\Sigma x]^2} \cdot \sqrt{N\Sigma y^2 - [\Sigma y]^2}}$$

Using the MonroeEpic calculator program, r was computed to be equal to 0.783.

APPENDIX 3A

LINEAR REGRESSION CURVE COMPUTATION

The example being used here is the data from the 0 hours post pulse sample from Figure 19.

If we consider "fraction number" to be the x ordinate, and "cpm/cell" to be the y ordinate, then a typical set of co-ordinates making up the linear regression curve can be defined as x_i, y_i. In the example below, n=4, where n is defined as the number of "points" on the curve.

x_i	x_i^2	y_i	xy
5	25	264	1320
6	36	331	1986
7	49	365	2555
8	64	406	3248
Σ=26	Σ=174	Σ=1366	Σ=9109

The slope of the linear regression curve, m, is computed based on the following formula:

$$m = \frac{n\Sigma xy - \Sigma x \Sigma y}{n\Sigma x^2 - [\Sigma x]^2}$$

Using the Monroe Epic calculator program, m was computed to be equal to 46. The units of the slope are "cpm/cell/fraction".

APPENDIX 3B

DETERMINATION OF WHETHER THE SLOPES OF ANY TWO LINEAR REGRESSION CURVES DIFFER AT THE 95% CONFIDENCE LEVEL

(personal communication, Dr. M. Haq)

Two hypotheses are being considered here. They can be represented by the following notations:

H_1: $m_1=m_2$

H_2: $m_1>m_2$

where m_1 is defined as the slope of the linear regression curve at the first sampling time, and m_2 is defined as the slope of the linear regression curve at the second sampling time

In other words, either the slopes of the linear regression curves, at two sampling times after pulsing, are equal (H_1), or the slope decreases with time into the chase (H_2).

The following computations are designed to determine which hypothesis is correct:

$$S_1^2 = \frac{1}{n_1-2} \left[\Sigma(yi-\bar{y}i)^2 - m_1^2 \Sigma(xi-\bar{x}i)^2\right] \quad \text{equation 1}$$

$$S_2^2 = \frac{1}{n_2-2} \left[\Sigma(yi-\bar{y}i)^2 - m_2^2 \Sigma(xi-\bar{x}i)^2\right] \quad \text{equation 2}$$

where: (a) S_1^2 and S_2^2 are defined as the variances for samples obtained at times 1 and 2 post pulse, respectively

(b) n_1 and n_2 are defined as the number of determinations ("points") made at times 1 and 2, resp.

APPENDIX 3B

(c) x_i and y_i are defined as in Appendix 3A

(d) the value enclosed within the [ ] brackets is often referred to as the "sum of squares", or "SS".

From equations 1 and 2 (above) an overall variance can be calculated. It is denoted as S_*^2.

$$S_*^2 = \frac{SS(1) + SS(2)}{n_1 + n_2 - 4}$$

By taking the square root of this number, we can arrive at a value for S_*.

Next we calculate a value for t, according to the formula:

$$t_{calc} = \frac{m_1 - m_2}{S_* \sqrt{\underbrace{\frac{1}{\Sigma(xi-\bar{x}i)^2}}_{\text{for first sample}} + \underbrace{\frac{1}{\Sigma(xi-\bar{x}i)^2}}_{\text{for second sample}}}}$$

Finally we compare this t value with $t_{.05}$ (right tail) for $(n_1 + n_2 - 4)$ degrees of freedom, from a Student's t table. If $t_{calc} > t_{.05}$, then we reject H_1 and accept H_2. If, on the other hand, $t_{calc} \leq t_{.05}$, then we can not accept H_2 with 95% confidence.

An example of how these calculations are done is found on the next page, for the 0 hours post pulse and 4 hours post pulse linear regression curve slopes, from the data of Figure 19.

APPENDIX 3B

0 hours post pulse (see Appendix 3A)

$\bar{x}_i = 6.5$

$\bar{y}_i = 342$

$n = 4$

$m = 46$

$x_i - \bar{x}_i$	$(x_i - \bar{x}_i)^2$	$y_i - \bar{y}_i$	$(y_i - \bar{y}_i)^2$
-1.5	2.25	-78	6084
-0.5	.25	-11	121
0.5	.25	23	529
1.5	2.25	64	4096
	$\Sigma = 5.00$		$\Sigma = 10830$

4 hours post pulse (see Appendix 3A)

$\bar{x}_i = 5.0$

$\bar{y}_i = 261$

$n = 5$

$m = 19$

$x_i - \bar{x}_i$	$(x_i - \bar{x}_i)^2$	$y_i - \bar{y}_i$	$(y_i - \bar{y}_i)^2$
-2	4	-54	2916
-1	1	- 2	4
0	0	- 7	49
1	1	44	1936
2	4	19	361
	$\Sigma = 10$		$\Sigma = 5266$

APPENDIX 3B

$$s_1^2 = \frac{1}{2}\ [10830 - 1849(5)]$$

$$s_2^2 = \frac{1}{3}\ [5266 - 361(10)]$$

$$s_*^2 = \frac{10830 - 1849(5) + 5266 - 361(10)}{5}$$

$$= 648$$

$$s_* = 25$$

$$t_{calc} = \frac{27}{25\sqrt{\frac{1}{5} + \frac{1}{10}}}$$

$$= 1.89$$

$t_{.05}$ for 5 degrees of freedom = 2.02

Since t_{calc} is not > $t_{.05}$, we can not reject hypothesis 1, and therefore must conclude that, at the 95% confidence limit, the slope value at 4 hours post pulse (19) is not significantly different than the slope value at 0 hours post pulse (46).

APPENDIX 4

CALCULATION OF THE DURATION OF EACH PHASE OF THE L-M CELL CYCLE

A) From the experiment described in Results section S, the following data was obtained:

1) % cells at 0 hours post pulse with silver grains (S phase cells) = 40

2) % mitotic cells with silver grains (M phase cells which have passed through S and G_2):

Time post pulse	%
4 hours	0
6 hours	0
8.5 hours	17
10 hours	17

3) Cell doubling time = 26 hours

4) Mitotic index of an untreated population = 0.17%

B) CALCULATIONS

1) According to Mitchison's formula (1971), $t_M = T \times M.I. \times 1.44$ where t_M is defined as the time for mitosis, M.I. is defined as the mitotic index (ranging in value from 0 to 1), and T is defined as the whole cycle time.

From the above formula it follows that $t_M = 26 \times .0017 \times 1.44$

$= 0.06$ hours

2) t_S, the time for S phase, is approximately equal to the proportion of cells which incorporate a pulse of labelled TdR, and hence in this case = (0.4 x 26) hours = 10.4 hours.

APPENDIX 4

3) t_{G_2}, the time for G_2 phase, is approximately equal to the time taken between the pulse and the appearance of significant numbers of labelled mitotic cells. From the data in A2) above, $8.5 < t_{G_2} > 6$. For simplicity we will take the value of 8 hours.

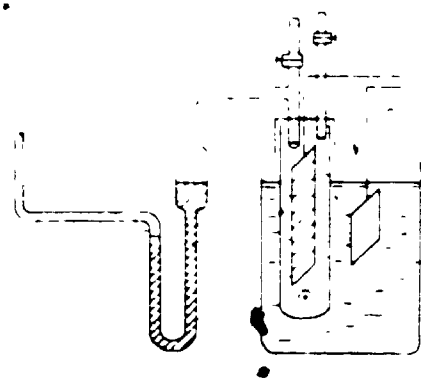
4) t_{G_1}, the time for G_1 phase $= T - (t_M + t_{G_2} + t_S)$

$= 26 - (0.06 + 8 + 10.4)$

$= 7.5$ hours, approximately.

APPENDIX 5

PRINCIPLE OF THE COULTER AND CELLOSCOPE ELECTRONIC CELL COUNTERS



THE COULTER PRINCIPLE

Operation is based on electrical condutivity difference between particles and common diluent. Particles act as insulators, diluents as good conductors. The particles, suspended in an electrolyte, are forced through a small aperture through which an electrical current path has been established. As each particle displaces the electrolyte in the aperture a pulse proportional to the particle volume is produced. Thus a 3 dimensional particle volume response is the basis for all sizing regardless of position or orientation of the particle in solution. Particle volume is the most accurate way to measure size.

APPENDIX 6

PRINCIPLE OF THE CYTOGRAF CELL SIZER

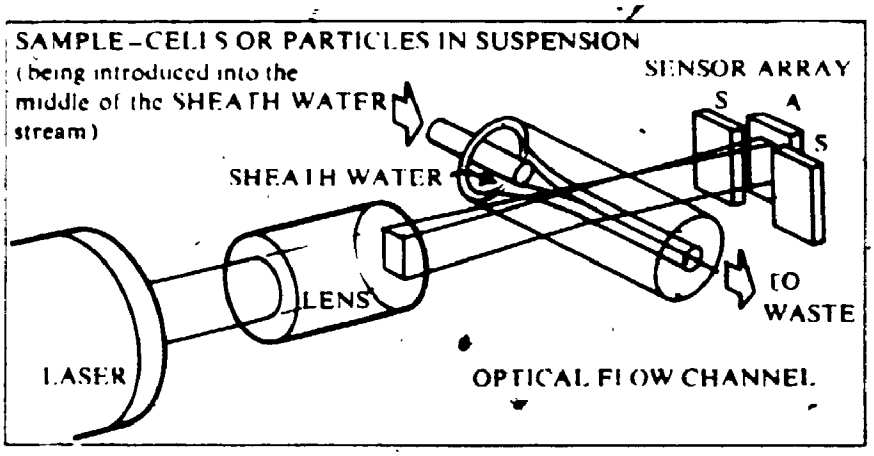


Fig. 1 Diagram of Instrument Optical System

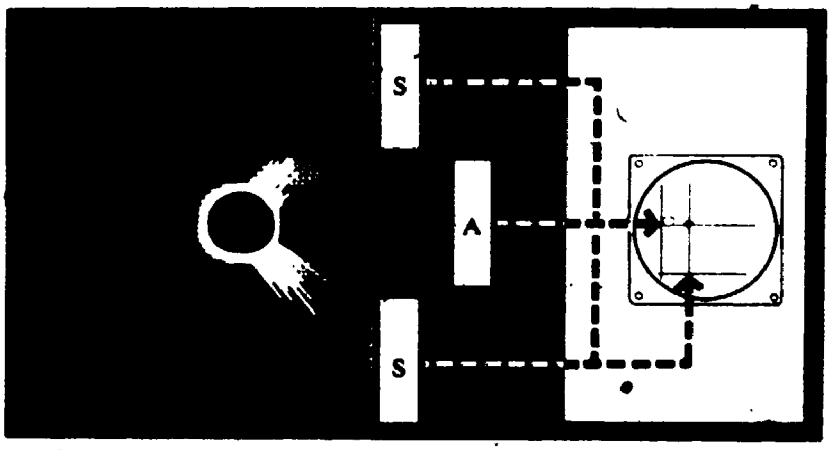


Fig. 2 Basic CYTOGRAF instrument function. Cell being individually illuminated. Light being measured by sensor array, and measurement displayed on oscillos as a dot.

A - the axial sensor. This sensor receives only the undiverted laser beam, and primarily measures diffracted light.

S - the forward scatter sensors. These sensors detect scattered light.

APPENDIX 7

RELATIONSHIP BETWEEN CELL VOLUME INCREASE AND CELL DENSITY DECREASE

To see if the 13% cell volume increase, which results by 3 hours PI when L-M cells are infected with vaccinia virus, is sufficient to account for the density decrease seen in Ficoll gradients, the following calculations were performed:

At 0 hr PI the main cell band is found at the 15-16% Ficoll interface. Taking 15.5% as the approximate concentration at this position, the density of Ficoll was calculated to be 1.0872 gm/cc. At 3 hr PI the main cell band is found at the 14-15% Ficoll interface, representing a density of 1.0861 gm/cc.

Thus the difference in density between these 2 banding positions = 1.0872 - 1.0861 = 0.0011 gm/cc. Expressed as a percent decrease, this is found to equal 0.1%. The question being asked then is whether the 13% volume increase can account for the 0.1% density decrease.

Assume that at 0 hr PI the cell volume = 100 μ^3. Then at 3 hr PI its volume = 113 μ. Visualize the cell as consisting of 2 sections, one (A) 100 μ^3 and one (B) 113μ^3 in volume. ρ_A = 1.0872; ρ_B is unknown, but as a MINIMUM = 1.0000. Therefore the overall minimum weight of the cell at 3 hr PI = 100 X 1.0872 + 13 X 1.0000 = 121.72 units. Hence the overall minimum density of this cell = $\frac{121.72}{113}$ = 1.0772 gm/cc. Thus if the 13% volume increase of cells after infection is due to uptake of water only, then the volume increase is (more than) sufficient to account for the density decrease. Even if ρ_B is significantly greater than 1.000, the overall density of the cell can still be shown to be less than 1.0861 gm/cc.

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